Oxidase-based biocatalytic processes

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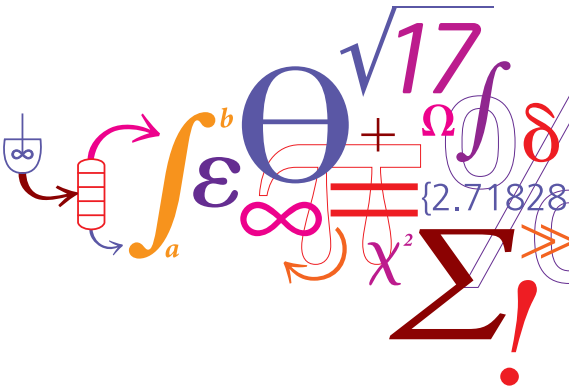

Hemalata Ramesh
Ph.D. Thesis
September 2014

DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

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PhD. Thesis

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Abstract

Biocatalytic processes are gaining significant focus in frontiers where they offer unique advantages (selectivity and mild operating conditions) over chemical catalysts. It is therefore not surprising that there have been many industrial biocatalytic processes implemented.

Despite past successes, the implementation of a new biocatalytic process still presents some challenges (demands placed on the biocatalyst) in terms of the requirements to make a viable industrial process. In order for a biocatalytic process to be economically successful, it is necessary that certain a set of target metrics (product titre, biocatalyst yield or space time yield and reaction yield) are achieved. Hence, the biocatalyst must be able to work at high substrate and product concentrations. Such constraints that arise from the biocatalyst are classified as biocatalyst-related limitations. In addition, other limitations can arise from the reaction species (substrate and product volatility for example) and the process (such as oxygen supply, ability to control pH) and are classified as reaction-related and process-related constraints respectively. Although the development of biocatalyst and process engineering tools offers a number of solutions to overcome the limitations, it is often complicated to identify the key limitation of the system that prevents economic scale-up. Hence, development of a systematic method for identifying the limitations during early-stage development of a biocatalytic process and potentially the order in which they need to be tackled would offer a valuable tool for process development.

Biocatalytic oxidations are potentially of great value because of the selective chemistry that they offer, resulting in higher yields compared to those achievable through chemical catalysis. Oxidases are particularly interesting biocatalysts because they use a mild oxidant (oxygen) as a substrate as opposed to their chemical counterparts which use strong oxidants such as permanganates. A class of oxidases called monoamine oxidases has been used as the central case study for the thesis. The rationale for choosing this system is that it has been shown to exhibit the potential for resolution of racemic amines, and is capable of producing industrially interesting imines which are rather difficult to synthesize by chemical routes.

An important aspect for biocatalytic reactions would be the implementation of monitoring and control systems that allow for rapid data collection to gain process knowledge. For oxidase-based biocatalysis, oxygen is consumed in stoichiometric amounts for the reaction. Therefore, oxygen sensors which can measure the oxygen concentration can be a valuable tool for monitoring of the process. The thesis exemplifies the use of novel solvent-resistant oxygen sensors as supporting technology for oxidase-based reactions using a glucose oxidase reaction system as an example.

Implementation of biocatalytic oxidation at scale still requires process knowledge which includes the limitations of the system and the knowledge about the potential solutions available to alleviate these limitations. This thesis presents a methodology for development of oxidase-based biocatalytic processes. A particularly important aspect of the methodology includes the use of *in silico* analysis where property prediction tools have been used to identify the potential limitations to the reaction system prior to experimentation. Such an analysis presents the opportunity to direct experimental work and therefore reduce the time and effort spent on process development, by eliminating unfeasible routes. The example chosen for the development of the methodology was a specific monoamine oxidase-based syntheses for the production of a pharmaceutical intermediate. This particular reaction system was chosen because of the potential use of the product of the biocatalytic reaction as a pharmaceutical intermediate. However, there was little information on the reaction system in the literature for the use of this biocatalyst for synthesis of chemicals. Therefore, early stage process understanding was required. The chapters of the thesis identify the potential limitations for the reaction system by systematic evaluation of the reaction system through the use of property prediction tools as well as experiments. The results obtained from the experiments are then used to identify the bottleneck for the implementation at scale. Furthermore, a discussion of the limitations and the order which they need to be tackled is presented.

Dansk Resumé

Fokus på biokatalytiske processer er øget betydeligt på grund af deres unikke fordele (selektivitet og milde reaktionsbetingelser) i forhold til kemiske katalysatorer. Det er derfor heller ingen overraskelse at mange biokatalytiske processer allerede er blevet implementeret industrielt.

Til trods for den hidtidige succes er det stadig en udfordring at sikre en bæredygtig industriel proces, når en ny biokatalytisk reaktion skal implementeres på industriel skala. Det er nødvendigt at en række mindstemål for processen (mht. produkt koncentration, biokatalysator udbytte, volumetrisk udbytte og reaktion udbytte) er opnået for at sikre økonomisk succes for en biokatalytisk proces. Det er derfor nødvendigt at en given biokatalysator fungere selv ved høje substrat- og produktkoncentrationer. Proces restriktioner der fremkommer på grund af biokatalysatoren er klassificeret som biokatalysator-relaterede begrænsninger. Ydermere kan der fremkomme begrænsninger grundet reaktions specier (for eksempel substrat og produkt flygtighed) samt selve processen (så som oxygen tilførsel eller evnen til at kontrollere pH), hvilke er klassificeret som henholdsvis reaktions- og proces-relaterede begrænsninger. Ingeniørværktøjer til forbedring af både biokatalysatorer og kemiske processer gør det muligt at afhjælpe system begrænsningerne, men det er oftest kompliceret at identificere nøgle-begrænsningerne der forhindre en økonomisk forsvarlig opskalering. Det er derfor nødvendigt at udvikle systematiske metoder der gør det muligt at identificere disse begrænsninger i en tidlig udviklingsfase af en biokatalytisk proces og potentielt også rækkefølgen som disse begrænsninger skal afhjælpes i.

Biokatalytiske oxidationer er potentielt meget værdifulde på grund af den høje kemiske selektivitet de tilbyder, hvilket resulterer i højere udbytte sammenlignet med det opnåeligt med kemiske katalysatorer. Oxidaser er særligt interessante biokatalysatorer, da de udnytter et mildt oxidationsmiddel (oxygen) som substrat modsat deres kemiske modstykker der bruger stærke oxidationsmidler så som permanganater. Monoaminoxidaser, en klasse af oxidaser, er i denne afhandling brugt som det primære case study. Rationalet for valget af netop dette system er at enzymklassen kan bruges til resolution af racemiske aminer samt industriel produktion af interessante iminer, som ellers er meget svære a syntetisere via kemiske ruter.

Implementering af måle- og kontrolsystemer der muliggør hurtig data indsamling vil være et vigtigt aspekt for biokatalytiske reaktioner. Oxidase-baseret biokatalyse forbruger oxygen i støkiometriskmængde. Oxygensensorer der kan måle oxygenkoncentrationer kan derfor udgøre et værdifuldt måleinstrument til at følge processen. Afhandlingen giver eksempler på brugen af moderne oxygensensorer der er

modstandsdygtige overfor organiske solventer der kan benyttes til at følge eksempelvis en glukoseoxidase katalyseret reaktion.

Implementeringen af biokatalytisk oxidation på industrielskala kræver proceskendskab som inkluderer systembegrænsninger og viden der gør det muligt afhjælpe disse begrænsninger. Denne afhandling præsenterer en metodologi for udviklingen af oxidase-baserede biokatalytiske processer. Et specielt vigtigt aspekt i metodologien inkluderer brugen af in silico analyse hvor værktøjer til forudsigelse af kemiske egenskaber er brugt til at identificere de potentielle systembegrænsninger før eksperimenter. En sådan analyse giver muligheder for at give retningslinjer for efterfølgende eksperimentelt arbejde og derved reducere mængden a tid og ressourcer brugt på procesudvikling ved at udelukke urealiserbare alternativer. En monoaminoxidase baseret syntese af et intermediært lægemiddel blev benyttet som eksempel i udviklingen af metodologien. Dette reaktionssystem blev valgt specifikt på grund af den potentielle anvendelse af produktet som et vigtigt intermediært lægemiddel. Informationer i den videnskabelige litteratur omkring brugen af denne biokatalysator og det pågældende reaktionssystemet var dog yderst begrænsede, hvilket gjorde tidlig procesforståelse uundværligt. Kapitlerne i denne afhandling identificerer de potentielle begrænsninger i reaktionssystemet ved hjælp af systematisk evaluering af systemet gennem brugen af værktøjer til forudsigelse af kemiske egenskaber samt eksperimenter. Resultaterne opnået gennem det eksperimentelle arbejde er derefter brugt til at identificere flaskehalsene for implementeringen af processen på industrielskala. Ydermere bliver systembegrænsninger og rækkefølgen de skal afhjælpes i diskuteret grundigt.

Abbreviations and Nomenclature

Abbreviation	Description
AAO	Amino acid oxidase
ACA	Amino cephalosporanic acid
AMBA	Alpha methylbenzyl amine
CE	Crude-enzyme
CPME	Cyclopentylethyl ether
DO	Dissolved oxygen
E.C.	Enzyme commission
E. coli	*Escherichia coli*
e.e.	enantiomeric excess
FAD	Flavin adenine dinucleotide
FDA	Food and drug administration
FDH	Formate dehydrogenase
FIA	Flow injection analysis
FMN	Flavin mononeucleotide
FTIR	Fourier transform spectroscopy
GC	Gas chromatography
gcdw	Gram cell dry weight
GMO	Genetically modified organism
GOx	Glucose oxidase
h	Hour(s)
HCV	Hepatitis C virus
ISPR	*In situ* product removal
ISSS	*In situ* substrate supply strategy
KPi	Potassium phosphate buffer
LC/MS	Liquid chromatography/mass spectrometry
LB	Luria Bertani
LED	Light emitting diode
M	Molar
MAO	Monoamine oxidase
MAO-N	Monoamine oxidase from *Aspergillus niger*
MTBE	methyl *tert*butyl ether
MTQ	1-methyl-1,2,3,4-tetrahydroisoquinoline
N.A.	Not applicable
N.D.	Not determined
NMR	Nuclear magnetic resonance
NIR	Near infrared
pET 16	Vector containing N-terminal His Tag and a T7 expression region
PQQ	Pyrroloquinoline quinone
rpm	Rotations per minute

TB	Terrific broth
TTN	Total turnover number
WC	Whole-cells
WT	Wild type

Nomenclature	**Description**	**Unit**
c_{crit}^{aq}	Critical concentration of inhibitor in aqueous phase	M
f_i	Constant (=25 for liquids at room temperature)	Dimensionless
LogP	Octanol-water partition coefficient	Dimensionless
$LogP_i$	Octanol-water partition coefficient of inhibitor	Dimensionless
OD_{600}	Optical density at wavelength of 600 nm	Dimensionless
pKa	Acid dissociation constant	Dimensionless
s_i^{aq}	Aqueous solubility of inhibitor	M
T	Temperature	˚C

Preface

The work presented in this thesis was prepared at the Department of Chemical and Biochemical Engineering (KT), at the Technical University of Denmark (DTU) in partial fulfilment of the requirements for the PhD. Degree in Engineering.

The work was conducted at the CAPEC-PROCESS center (former Centre for Process Engineering and Technology, PROCESS, DTU Chemical Engineering) from October 2011 to September 2014. The project was supervised by Prof. John M. Woodley and Dr. Ulrich Krühne.

The project was partially funded by BIONEXGEN, financed by the European Union through the 7th Framework people Programme (grant agreement no.: 266025). Collaborations were extended to other Universities and industries (in particular Technical University of Slovakia, University College of London and CLEA Technologies) in the framework.

Kgs. Lyngby,

September 2014

Hemalata Ramesh

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1. Thesis Introduction

1.1 Background

Chemical industries have been producing value-added products which include a plethora of chemicals (which can broadly be classified into pharmaceuticals, fine chemicals, speciality chemicals and bulk chemicals). The need for such chemicals combined with the realisation of the impact of civilization on the environment has driven chemical industries towards manufacturing such commodities through green chemistry. To this effect, the first principles of green chemistry were promoted by Anastas and Warner[1]. Green chemistry, in principle consists of processes that are considered to use raw materials (preferably renewable) effectively, generate less waste, and do not use toxic/hazardous materials in the production[2]. Consequently, bioprocesses (consisting of fermentation and biocatalysis) that use renewable materials and specific catalysts to produce the target molecule have seen rapid development in the past few years.

Fermentation involves growing of cells and using their innate metabolic pathway to produce the product of interest (Figure 1-1). The products could include enzymes, peptides, feed for animals and chemicals such as propane diol, butanol, succinic acid, citric acid among others. Fermentation has been used to produce cells which contain the enzyme of interest; however, fermentation is not in the scope of the thesis and will not be discussed in detail.

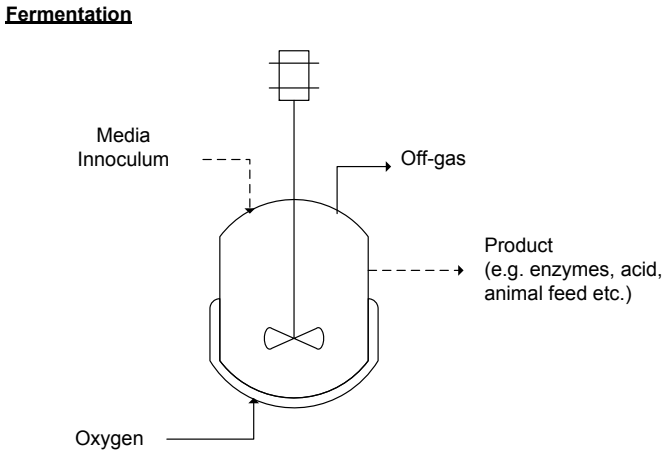


Figure 1-1: Schematic representation of batch fermentation.

Government policies and public pressure have contributed to the adoption of biocatalysis for greener manufacture[3]. Biocatalysis is a class of bioprocesses that are based on the use of enzyme(s) (either in free or immobilized forms) or whole-cells (growing or resting cells) as a catalyst[4,5]. In Figure 1-2 biocatalysis using extracellular enzyme has been depicted. The focus of the thesis will be on biocatalytic processes.

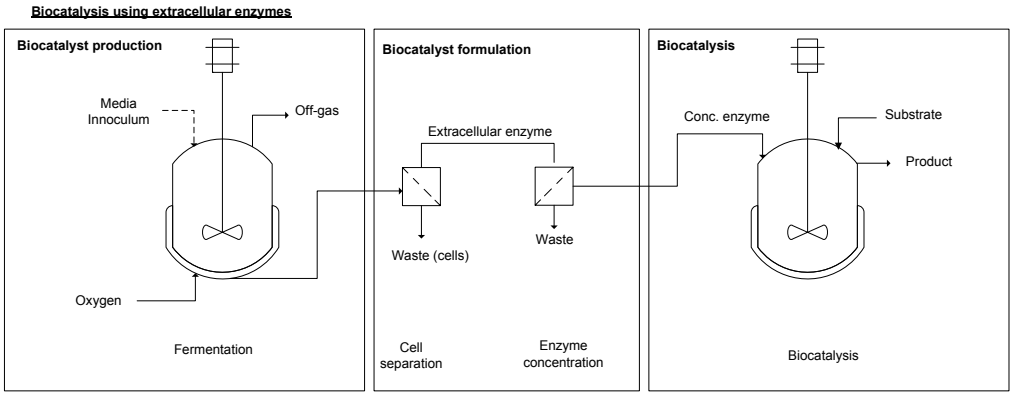


Figure 1-2: Biocatalysis with an extracellular enzyme.

1.2 When to use biocatalysis for chemical synthesis

Selectivity is an important trait for catalysts employed in the production of chemicals. A catalyst which can be highly selective ensures a higher yield compared to a non-selective process (because a low selectivity would result in the loss of substrate by converting them into other undesirable products). Reactions catalysed with chemical catalysts often suffer from low selectivity, especially when chiral molecules are being produced. In order to selectively add a functional group using a chemical catalyst, the substrate molecule is often protected prior to the reaction and de-protected after the desired functional group has been added[6]. These additional steps involve some losses in the reaction yield (i.e. gram of product produced per gram of substrate consumed) and generate more waste, thus going against green chemistry principles. Biocatalysis has gained importance in part due to their selectivity (regio- and stereo-selectivity) and the unique chemistry (compared to their chemical counterparts) that they offer[7]. Due to the highly selective nature of the biocatalysts, biocatalysis offers a much greater yield/atom economy over conventional chemistry.

Enzymes in general work very well at temperatures close to the natural environment of the organism from which the enzymes are isolated[8]. Because of this, it is not surprising that these catalysts are active at temperatures around 30 ^{o}C. Biocatalysts thus additionally offer the advantage of operating at fairly low temperatures. Operating at low temperatures with high catalytic activity (which can easily be achieved through biocatalysis) can come in handy when using substrates that are less stable at high temperature. One such substrate that has benefited from biocatalysis is docosahexaenoic acid which is prone to oxidation[9].

Furthermore, enzymatic systems can operate well in aqueous media making the use of organic solvents unnecessary. However, since they generally operate in dilute systems, the requirement of downstream processing and the energy costs that come with it needs to be considered. Hence, operating at aqueous environment doesn't necessarily make the process green. However, in case of reactions where oxygen or gases need to be supplied, use of organic solvents can cause handling difficulties and explosion hazards. In such cases, working in aqueous media can prove to be advantageous.

Owing to these advantages it is not surprising that biocatalytic reactions not only have a major importance in the pharmaceutical industry but have also been extended to other areas of the chemical industry that include fine and bulk chemistry[10-13]. Biocatalytic reactions using hydrolases and transaminases have been established over the past few years[7,14-17]. Current focus has been shifted to employing enzymes capable of catalysing oxidation-reduction reactions and formation of C-X or C-C bonds. This is because carbon-carbon and carbon-functional group bond formation offers the backbone for generating several organic molecules. However, use of conventional chemistry for formation of C-C or C-X bonds can often be difficult and involve the use of additional agents for protecting and de-protecting the functional groups. Therefore, avoiding these agents and producing the chemical intermediate of interest has potential. Thus, enzymatic reactions have been identified and consequently there has been an increase in enzyme development (to cope with the conditions required for the application of the biocatalyst for synthesis) that has allowed for novel C-C, C-X bond formations through biocatalysis[18,19].

Of these reactions, oxidations are particularly interesting because they form the cornerstone for transformation in organic chemistry. Oxidation reactions offer access to different functional groups and can therefore offer functionalization of building blocks used in synthesis. Although the focus on green chemistry has moved traditional chemical oxidations from being catalysed by toxic catalysts, such as chromates, towards the use of milder transitional metal catalysts, the chemical route still suffer from issues of low selectivity[20,21]. The low yield and selectivity obtained from chemical catalysis combined with the high

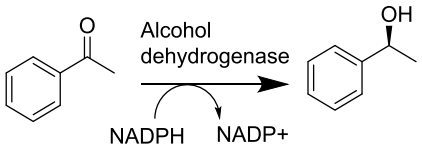
selectivity offered from biocatalysts has caused a paradigm shift towards the use of biocatalytic oxidations[22]. Industrial implementations of biocatalytic oxidations are now available[23,24]

Biocatalytic oxidations will be discussed further in this chapter leading up to the scope of the thesis.

1.3 Biocatalytic oxidations

Oxidizing enzymes can be broadly classified as dehydrogenases (EC 1.1.1.x), oxygenases (EC 1.13.x.x), oxidases (EC 1.1.3.x) and peroxidases (EC 1.11.1.x). Oxygenases are a class of enzymes that use oxygen as a substrate and introduce one or two oxygen molecules in the substrate[25,26]. Peroxidases use hydrogen peroxide as electron acceptor and produces water as a product[27]. Oxidation reactions, in principle, are also accessible by alcohol and amino acid dehydrogenases[28]. However, these enzymes require expensive and often unstable cofactor NADH. Additionally, co-factor regeneration systems need to be employed[29,30]. An example of alcohol dehydrogenase catalysed reaction has been depicted in Scheme 1-1.

Oxidations of alcohols and amines represent two important oxidation reactions in synthetic chemistry and both these reactions can be catalysed by oxidases[24]. Oxidases are a class of enzyme which uses oxygen as a terminal electron acceptor for catalysing the oxidation of the substrate molecule[24]. The major focus of this thesis is on biocatalytic oxidations catalysed by oxidases.

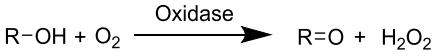


Scheme 1-1: Example of alcohol dehydrogenase catalysed reaction.

Alcohol and amine oxidases, which catalyse C-O and C-N oxidations, respectively, and depend on metal or flavin groups for catalytic activity, have consequently become an important class of biocatalytic oxidations. Therefore, biocatalytic oxidations of alcohols and amines are gaining focus for chemical synthesis and some examples will be discussed in the following sections. Of these oxidations, oxidations of amines are very difficult using chemical synthesis and more easily accessed by biocatalysis. Therefore, they are the central goal of the thesis.

1.3.1 Alcohol oxidases

Alcohol oxidases (E.C. 1.1.3.x) catalyse the oxidation of alcohols by using molecular oxygen as electron acceptor and produce hydrogen peroxide (Scheme 1-2). This class of enzyme can further be classified as primary and secondary alcohol oxidases.

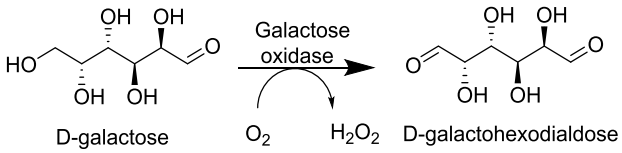


Scheme 1-2: Alcohol oxidation catalysed by alcohol oxidases.

Primary alcohol oxidases

Primary alcohol oxidases can catalyse the oxidation of aliphatic or aromatic alcohols. Aliphatic alcohol oxidases (eg. from *Candida boidinii*) have been used to catalyse the oxidation of primary alkanols to the aldehyde to generate hydrogen peroxide[31-34]. Oxidation of racemic mixtures to obtain optically pure aldehyde have been achieved[35].

A significant example of a primary alcohol oxidase is the galactose oxidase which converts the sugar galactose to galactohexodialdose (Scheme 1-3). Wild type enzyme is highly selective to D-galactose and D-talose[36]. However the substrate spectra has been increased towards other sugars such as glucose and fructose through protein engineering[37]. Galactose oxidase has found applications in sensor development[38] as well as in synthetic chemistry.



Scheme 1-3: Conversion of galactose by galactose oxidase.

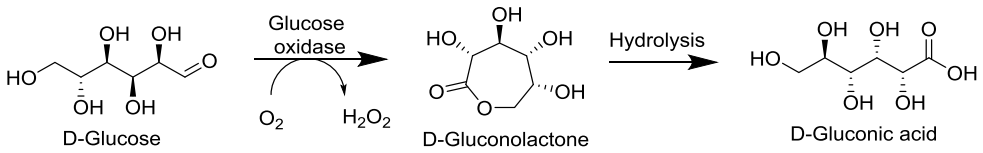
Application of galactose oxidase in synthesis includes stereospecific oxidation of glycerol[39], kinetic resolution of D-threose and the conversion of 3-halo-1,2- propane diols to the corresponding aldehyde. However, preparative-scale oxidations with galactose oxidase is slow and often results in low yields[29].

Secondary alcohol oxidases

Secondary alcohol oxidases include the most studied catalyst of the oxidase family - glucose oxidase. Other oxidases that fall in this group would include pyranose oxidase, glycolate oxidase and cholesterol oxidase.

Glucose oxidase

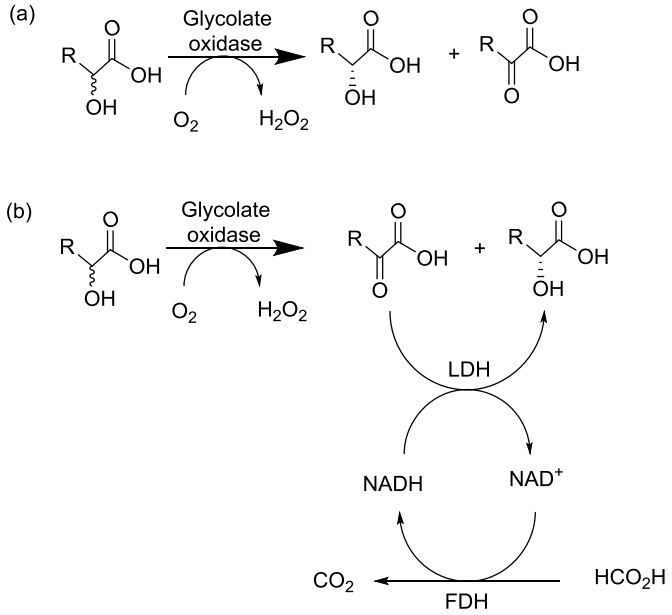
Glucose oxidase is used for conversion of glucose to gluconolactone which spontaneous hydrolyses to gluconic acid (Scheme 1-4). The reaction scheme is depicted in Scheme 1-4. The enzyme has been used widely in the food industry for making bread and for development of sensors[40,41]. The high substrate specificity towards glucose has made the application scope for this biocatalyst rather limited[29,42]. However, the enzyme has been widely studied and often used as a model system to represent the oxidase family.



Scheme 1-4: Oxidation of glucose by glucose oxidase.

Glycolate oxidase

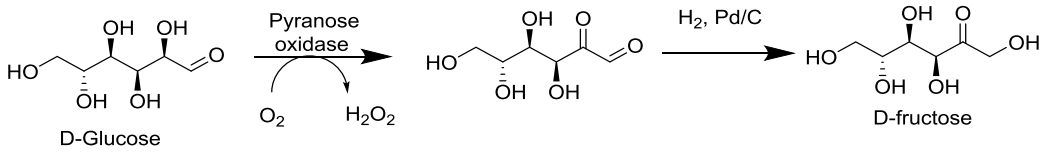
This class of oxidases is dependent on flavin mononeucleotide (FMN) and converts glycolic acid to glyoxylic acid. The biocatalyst has been employed for oxidation of 2-hydroxy acids to 2-keto acids and for deracemization of 2-hydroxy acids by using the enzyme in combination lactate dehydrogenase and formate dehydrogenase[43] (Scheme 1-5).



Scheme 1-5: (a) Oxidation by glycolate oxidase (b) Use of glycolate oxidase/lactate dehydrogenase (LDH) and formate dehydrogenase (FDH) for the production of optically pure dehydroxy acid (Adopted from Kroutil et. al.[29]).

Pyranose oxidase

Pyranose oxidase converts glucose into its corresponding ketose (the oxidation occurs at the C-2 position). The enzyme has been applied synthetically for the production of optically pure fructose from glucose as well as in the production of 5-keto-D-fructose[44,45]. From a clinical perspective, it has been used for the detection of glucitol in diabetic patients (glucitol is a marker for glycemia)[46].



Scheme 1-6: Production of D-fructose by chemo-biocatalysis.

1.3.2 Amine oxidases

Amine oxidases catalyse the oxidation of amines (primary, secondary or tertiary) to their corresponding aldehydes (in case of conversion of terminal amines) or ketones. These reactions are catalysed by flavin dependent oxidases. These conversions are central to synthetic chemistry because the conventional traditional routes to achieve C-N oxidations are, if not impossible, at least very difficult. These oxidations require rather aggressive oxidants and consequently suffer from low selectivity[24]. These challenges faced by chemical catalysis provide opportunities for biocatalytic C-N bond oxidations. Therefore, amine oxidase was chosen to form the central focus for this thesis. Therefore, a separate chapter on amine oxidases, in particular monoamine oxidase is dedicated for this system (Chapter 2).

1.4 Scope of the thesis

Oxidase-based biocatalysis as mentioned previously is gaining significant focus due their selectivity as well as the access they provide to oxidation reactions that are difficult to achieve using chemical synthesis. Implementation of biocatalytic process at scale presents some challenges and process development is often not straightforward. Therefore, it could be a valuable tool to arrive at a systematic methodology for process development. Hence, this thesis, in part, was aimed at characterising an oxidase-based system in a systematic manner and using the characterization to make suggestions for process development.

For developing a methodology for process characterisation of an oxidase-based process an amine oxidase (monoamine oxidase) based reaction system has been selected as the case study. The product of this reaction provided access to a pharmaceutical intermediate that potentially increases the yield by decreasing number of steps involved in the production of the drug molecule. However, there are only a few publications (patents and journal articles) available for the use this biocatalyst in synthetic route (most focus in the past decade has been on this particular biocatalyst for deracemization of amines). Because relatively few studies are available for using this enzyme in a synthetic route, the studies conducted focus on several early stage characterisation experiments for process development. The characterisation involved the use of property prediction tools and literature review for identifying potential limitations to the system. Following this, process characterisation was carried out through experiments with the purpose of quantifying the limitations that were identified through literature. Furthermore, process limitations that prevent economic feasibility of the reaction were identified and improvements were suggested to enable the system to reach the economic targets.

The second goal of the thesis involved the implementation of monitoring tools. Implementation of tools for monitoring allows for rapid data collection and thereby contributes to rapid acquisition of process knowledge. To this end, oxygen sensors were tested for their applicability. Novel oxygen sensors that were capable of working in organic solvents were tested for this purpose. Also since it was desired to use a well-characterised system for demonstrating the applicability of these sensors, a glucose oxidase based-reaction system was selected.

1.5 Structure of the thesis

The thesis is divided into 12 Chapters, which are grouped into 5 main parts. Part I, containing chapters 1-3 form introduction to the thesis, the case study (which focuses on biocatalysis by monoamine oxidase) and an introduction to oxygen sensors as supporting tools for biocatalytic oxidations. In Part II (consisting of Chapter 4), the applicability of oxygen sensors as tools for oxidation reactions has been established. In Part III made up of chapters 5-7 the analysis of potential limitations to the system and experimental work to quantify these limitations are presented. Part IV describes more experimental work to identify the key limitations of the reaction system, the order of the limitations and the implications for scale-up. Finally, the last 3 chapters (Part V) form the discussion, conclusion and future perspectives.

The different chapters can be split into 5 parts.

Part I - Background

The first part (Chapters 1, 2, 3) forms the background for oxidative biocatalysis and involves the use of literature to identify potential challenges for the target systems.

Chapter 1 provides an introduction and background to biocatalysis and oxidase based biocatalytic processes. It also presents the scope of this thesis.

Chapter 2 deals with the introduction to amine oxidases and biocatalytic reactions catalysed by amine oxidases have been presented. Furthermore, the motivation for selection of the said case has been presented.

Chapter 3 describes the use of oxygen sensors as tools for operating oxidase-based biocatalytic processes. A review of the most commonly used applications of advanced oxygen sensors has been presented.

Part II – Oxygen sensors as supporting tools for oxidase-based biocatalysis

The second part consists of chapter 4, where supporting technologies for oxidation reactions are explained. Here, the use of solvent resistant sensors for Kla measurements have been demonstrated using a glucose oxidase catalysed reaction system. The results presented in this chapter along with results from sensor calibration in collaboration with TU Graz and PyroScience have made the basis for a manuscript.

Part III – Analysis of process limitations of monoamine oxidase based biocatalytic process

The third part (Chapters 5-9) focuses on the characterization of a biocatalytic oxidation reaction which involve experimental validations of the limitations identified in system in part I of the thesis.

In Chapter 5, literature review has been presented which help identify the potential challenges for the target amine oxidase processes. Following these identifications, a list of potential solutions for corresponding challenges has been identified.

Chapter 6 focuses on the limitations that are arising from the reaction species that is the substrate and the product. This includes issues with pH shift caused due to the product, mass balances of the reaction and product toxicity. The chapter further introduces an ISPR technique to cope with the toxicity.

In Chapter 7, biocatalyst-related limitations which include interactions of reaction species with the biocatalyst (for example substrate and (co-)product inhibition and toxicity) have been identified and quantified. This chapter also deals with test for oxygen limitations for the system. Here, oxygen requirement for the target reaction system has been discussed. Major part of the results from this chapter is reported in Paper I.

Part IV – Analysis of process limitations for implementation at scale

The fourth part (consisting of Chapter 8 and 9) contains more experimental work that has been conducted to analyse the various process limitations of the target system and their implications for implementation of the reaction system at scale.

Chapter 8 presents an introduction to important process metrics for successful implementation of biocatalytic reactions. This further presents the interactions between process metrics and presents the effect of overcoming process limitations.

Chapter 9 deals with the handling of biocatalyst downstream of fermentation prior to biocatalysis. This chapter forms an extension of Chapter 5 in terms of the limitation arising from the handling of the biocatalyst. The results in this chapter with additional results from another partner from the EU project consortium are presented in paper II which is in preparation for submission.

Part V – General discussion and conclusions

The fifth part (Chapter 10-12) present the general discussion and conclusions from the work presented in the thesis. Future directions for the work are presented in the last chapter.

Chapter 10 presents general discussions on the two case studies. A methodology that was established for process characterization.

Chapter 11 lists the most significant conclusions from the work are stated.

In Chapter 12**,** few directions for future work and directions following the work in this thesis have been discussed in this chapter.

1.6 Contributions

Results from several stages of the project were presented in conferences and some of the experimental work has been published as manuscripts. The feedback obtained at these conferences and EU meetings greatly enriched the project's outcome. Besides the mentioned publications and conference presentations, bi-annual presentations were made at internal BIONEXGEN meeting with the collaborating partners and additional work-package meetings that were held during the course of the project.

Journal articles

The following journal articles were drafted from parts of this thesis.

Published

Ramesh, H., Woodley, J.M. (2014) Process characterization of a monoamine oxidase. J. Mol. Biocat. B, *106:124-131*.

This paper is based on the material presented in Chapter 7.

In preparation

Ramesh, H., Zajkoksa, P.; Rebros, M.; Woodley, J.M.; biocatalyst washing paper

This paper is based on material presented in Chapter 9.

Ramesh, H., Mayr T., Krühne U., Woodley J.M.; Use of oxygen sensors as tools for oxidase-based biocatalytic processes.

This paper forms the basis for discussion in Chapter 4.

Part I

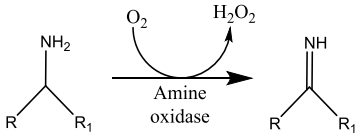
2. Introduction to the target reaction system

The previous chapter introduced oxidation reactions and further the importance of role of C-N bond oxidation through biocatalysis. Often, the oxidation of C-N bond is difficult to achieve through conventional chemistry and biocatalysis provides a valuable alternative. In this chapter, amine oxidases will be introduced and further the motivation for the target reaction system presented.

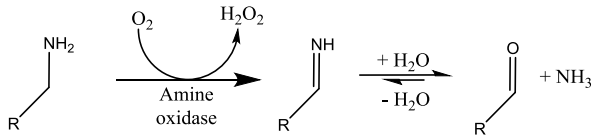
2.1 Amine oxidases

Amine oxidases (EC. 1.4.3.x) form a class of enzymes from the oxidoreductase family that catalyses the oxidation of amines. Amine oxidases are found in a wide range of organisms (consisting of both prokaryotes and eukaryotes). While they are found in the mitochondria in eukaryotes and are membrane bound, in prokaryotes they are not membrane bound. The primary function of the amine oxidase is to provide the prokaryotic organism with nitrogen by breaking down amines to form ammonia.

As with the other enzymes in the oxidase family, amine oxidases do not incorporate molecular oxygen in the target molecule but instead use oxygen as a terminal electron acceptor in the oxidation of an amine to an imine. The by-product of this reaction is hydrogen peroxide[47]. The general reaction scheme (corresponding to conversion of primary amines) for amine oxidases is shown in Scheme 2-1. In the case of primary amines, the imine product that is formed is often hydrolysed to form the corresponding aldehyde or ketone depending on the position of the amine group. Scheme 2-2 represents the oxidation of a primary terminal amine to an aldehyde.



Scheme 2-1: Amine oxidase catalysed oxidation of primary amines.



Scheme 2-2: Hydrolysis of primary amine to an aldehyde.

2.1.1 Types of amine oxidases

Two types of amine oxidases have been identified viz. type I and type II amine oxidase. Type I amine oxidases are copper containing enzymes that additionally require a co-factor (such as pyrroloquinoline quinone (PQQ)) for the activity of the enzyme. This class of enzyme is prevalent in bacteria, fungi and plants. The use of this class of enzyme for biocatalysis is limited - there is only one documented application for this system[48]. The authors reported a low E value (E=15) for this process and suggested that this example could be used as a basis for future biocatalyst development. However, a previous study on the catalytic mechanism of this enzyme suggests that an imine can covalently bind to the active site of the enzyme[49]. This phenomenon will cause irreversible inhibition of the enzyme making successful implementation in biocatalytic applications difficult. Such irreversible binding of molecules to enzymes is not uncommon to enzymatic reactions, and the molecules are generally referred to as inactivating agents.

For biocatalytic applications, significant focus (over the past decade) has therefore been on the latter type of amine oxidase (type II). Type II amine oxidases are flavin-dependent enzymes (use flavin adenine dinucleotide (FAD) as a co-factor). FAD participates in the catalytic conversion of the substrate to the product and is tightly bound to the active site of this enzyme, making the biocatalyst more favourable for synthetic purposes. The FAD acts as a redox partner by either accepting an electron or donating it to generate a free imine molecule.

One of the important enzymes of type II amine oxidase is a monoamine oxidase, which has shown promise in several industrial applications. The mechanism of the conversion of amine to imine by monoamine oxidase A and B has been discussed by Millers and Edmondson[50] (Figure 2-1).

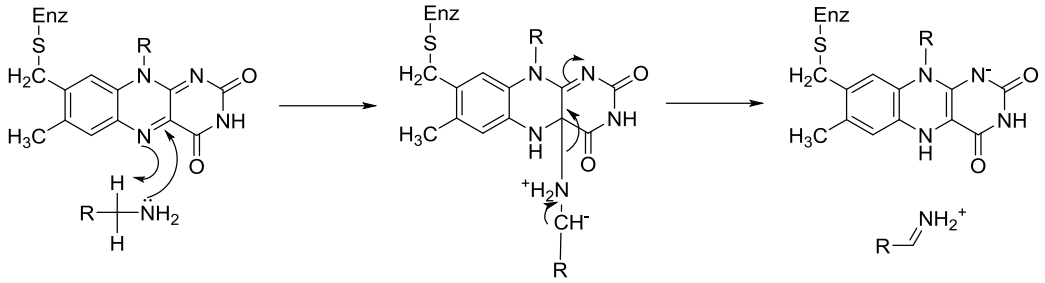


Figure 2-1: Mechanism of conversion of a primary amine by MAO. (Adopted from Miller & Edmondson, 1999[50]).

2.2 Monoamine oxidase-N (MAO-N)

Lerch and Schilling were the first to identify MAO-N in *Aspergillus niger* about 2 decades ago[51]. Following identification, the enzyme was cloned into *E. coli* through a pET3 vector containing a T7 promotor enabling the formation of soluble protein inside the cells[52]. Later, MAO-N was identified as a soluble protein and we have confirmed that the protein is constitutive (Appendix III). This development opened up the possibility to using this enzyme for biocatalytic applications. Simple amines (e.g. butlyamines) have been reported as the preferred substrate for the wild type enzyme[53]. To enable the use of the oxidase as a synthetic catalyst (of industrial relevance), further development of the biocatalyst was required (including improved applicability towards industrially relevant amines). Turner and co-workers have successfully combined random mutagenesis together with a rapid screening strategy to select mutants of interest [54]. While the initial mutants were developed to improve the spectra for primary amines[28], several rounds of development led to the usability of MAO-N for more complex amine molecules, including secondary and tertiary amines[55-60]. A list of the amines that have been used and the corresponding k_{cat} values (when available) have been listed in Table 2-1. It is noteworthy that the k_{cat} values reported in literature for the same mutant is quite different – sometimes as much as an order of magnitude of difference is seen (for example in the case of L-AMBA). Such differences could arise from the differences in the experimental method adopted for k_{cat} measurements. Nevertheless, these k_{cat} values can be used to show the substrate spectra has been improved and one can infer that the catalyst has a high specific activity. These developments have opened up the possibility of applying this class of enzyme for industrially relevant reactions. Examples of the use of monoamine oxidase in the past years will be discussed in the following section.

Table 2-1: Substrate spectra for MAO-N and the mutants.

Substrate	Wild Type/ Mutant	Mutation detail (if available)	kcat (s^{-1})
Amylamine	WT	N.A.	445[51]
	WT	N.A.	81[53]
	WT	N.A.	16.7[28]
	Mutant	Asn336Ser	2[28]
	Mutant	Asn336Ser	1.2[62]
	Mutant	Asn336Ser/Ile246Met	1.21[62]
L-AMBA	WT	N.A.	0.03[28]
	Mutant	Asn336Ser	0.1[28]
	Mutant	Asn336Ser	2.5[33]
	Mutant	Asn336Ser/Ile246Met	2.13[33]
D-AMBA	WT	N.A.	$1.6*10^{-4}$ [28]
	Mutant	Asn336Ser	0.001[28]
Benzylamine	WT	N.A.	199[51]
	WT	N.A.	23.3[53]
	WT	N.A.	6.2[28]
	Mutant	Asn336Ser	3.2[28]
Crispine A	Mutant	Ile246Met/Asn336Ser/Met348Lys/ thr384Asn/Asp385Ser	not available[56]
1-Cyclohexyl-N-methoxyethanamine	Mutant	not available	10.2[61]
S- 1-MTQ	Mutant	Asn336Ser	1.1[62]
	Mutant	Asn336Ser/Ile246Met	6[62]
R-1-MTQ	Mutant	Asn336Ser	0.06[62]
	Mutant	Asn336Ser/Ile246Met	0.08[62]
2-phenylpyrrolidin	Mutant	Asn336Ser	6.01[62]
	Mutant	Asn336Ser/Ile246Met	7[62]
4-chloro-benzhydrylamine	Mutant	Ile246Met/Asn336Ser/Met348Lys/ thr384Asn/Asp385Ser/Trp430Gly/ Phe210Met/Leu213Thr/Met242Gln /Ile246thr	not available[58]
1-phenyl tetrahydroisoquinoline	Mutant	Ile246Met/Asn336Ser/Met348Lys/ thr384Asn/Asp385Ser/Trp430Gly/ Phe210Met/Leu213Thr/Met242Gln /Ile246thr	not available[58]

2.2.1 MAO-N structure

MAO-N was first crystallised by Atkin and co-workers in 2008 which opened up the possibility of studying the structure and it's implication for biocatalysis[63]. Wild type MAO-N and two different mutants were successfully crystallized and reported in this paper. While the wild type enzyme is a tetramer in solution with a molecular weight of approx. 55 kDa, MAO-N-3 and MAO- N-5 exist as two tetramers and a dimer respectively. The crystallization consequently helped in decoding the presence of two channels (hydrophobic in nature) leading to the active site containing the FAD. The volume of these channels dictates the size of the substrate molecules that can enter the active site and consequently the substrate spectra of the biocatalyst. MAO-N-5 structural studies also indicate that each monomer contains 495 amino acid residues. The co-factor FAD is found to be in the oxidised form and held in the active site by non-covalent bonding[64]. The implication of a tightly bound FAD to the enzyme is that the biocatalyst will be catalytically active outside the cell membrane without additional cofactor being added to facilitate the reaction. This opens up the possibility of using this enzyme as a crude extract or in the purified form in an economical way.

2.3 MAO-N Application

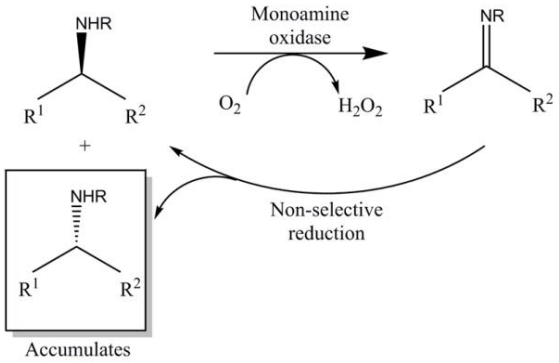
Once the substrate spectra for MAO-N was increased, its potential has been explored for three different purposes. The stereo-selectivity of the biocatalyst has been exploited for (i) dynamic resolution of racemic mixtures, (ii) synthesis of aldehydes from amines and (iii) synthesis of optically pure oxidation product (imine). The reason for using pure stereoisomer is that the regulatory authorities have mandated that pharmaceutical industry use optically pure compounds as drug molecules. All three reactions are discussed in the following section.

2.3.1 Synthesis of optically pure amines using MAO

Regulatory agencies such as FDA and consequently the pharmaceutical industry have been moving away from using racemic mixtures as drugs to using an optically pure compound. The reason for this is that only one of the enantiomer is usually the active drug component. The other enantiomer is either pharmacologically inert or could even have undesirable properties[65].

Turner and co-workers first established the deracemization reaction involving MAO-N in the year 2000. MAO-N has been used for the separation of a single stereoisomer from the racemic mixture by using the biocatalyst to oxidize one of the racemates to its corresponding imine. Following the oxidation step, a non-

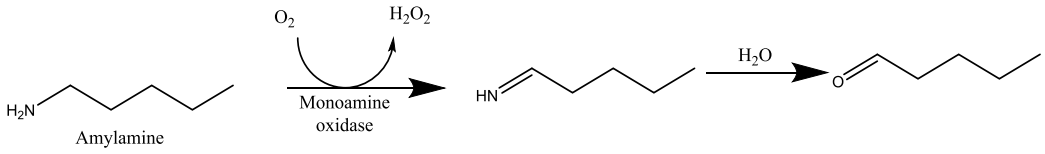
specific chemical reduction (using a chemical catalyst) is carried out to convert the imine into the racemic mixture. These steps are carried out for several cycles until one racemate is concentrated. Scheme 2-3 represents the general deracemization reaction. MAO-N can catalyse deracemization of several amines, some of which are depicted in Scheme 2-5 (Adapted from[56,58,62]).



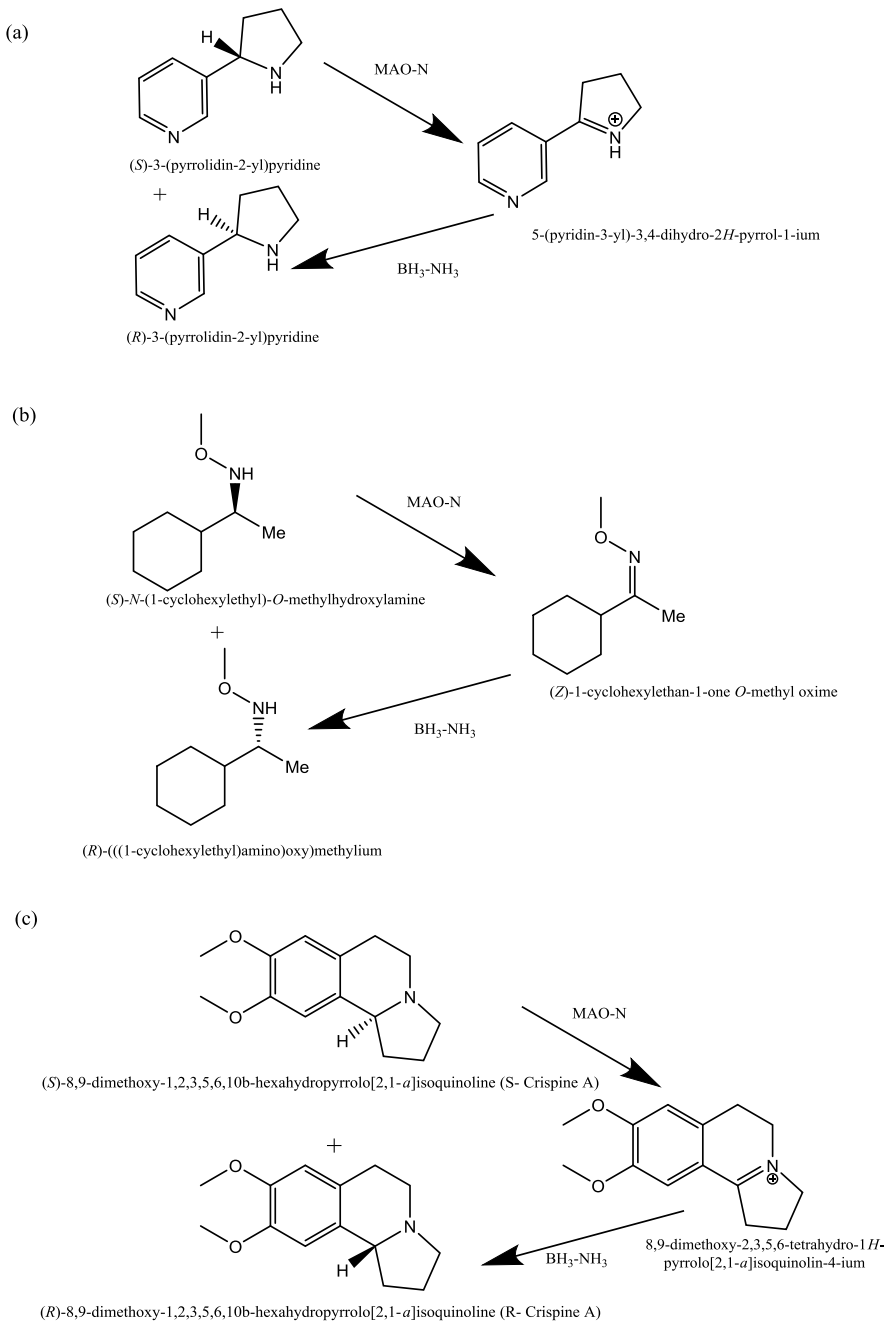
Scheme 2-3: **Deracemization using MAO.**

2.3.2 Synthesis of aldehyde from amines

Synthesis of aldehydes from amines is carried out in two steps, the first of which is catalysed by MAO. Following the conversion of primary amines to imines, the imine hydrolyses (spontaneously) to the corresponding aldehyde (Scheme 2-4). Therefore, this type of conversion is limited to primary amines because secondary and tertiary imines are less prone to hydrolysis. This type of reaction can be catalysed by wild type enzyme.



Scheme 2-4: Conversion of amine to aldehyde.



Scheme 2-5: Deracemization of (a) nicotine, (b) hydroxylamine and (c) Crispine A catalysed by different mutants of MAO-N (Adapted from[56,58,62]).

2.3.3 Synthesis of imine from amines

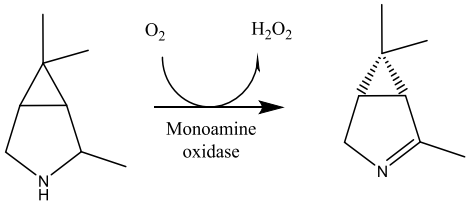
As mentioned earlier, MAO has been extensively used for deracemization reactions. However, several competing biocatalytic technologies (eg. using transaminases, lipases, imine reductases) are available for the synthesis of an optically pure compound[15,66,67]. Hence, the true potential of a monoamine oxidase lies in its application towards synthesis, rather than deracemization. Herein, the selectivity of the biocatalyst can be exploited to produce the target imine in a single step, which would otherwise require multiple steps using synthetic chemistry. The use of MAO in synthetic chemistry was first established by Köhler and co-workers, who used MAO for the synthesis of pyrroline residues[57]. Subsequently, the authors identified that the pyrroline residue produced could be used for the synthesis of drugs for the treatment of Hepatitis C.

A recent study reported that around 200 million people are affected by Hepatitis C. Of these, some of them do not respond to the treatment available today due to mutated virus and/or adverse effects of the drug. As a result, new drugs have been identified and recently have got FDA approval. Following this, two industrially relevant examples have been identified for production of drug intermediates (for the treatment of Hepatitis C) using MAO as the biocatalyst[68,69]. One of these examples is implemented in a joint work by Merck and Codexis which is discussed further.

Boceprevir intermediate

The first industrial example is from a recent joint publication from Merck and Codexis for the production of Boceprevir intermediate, which is a drug used for the treatment of Hepatitis C patients with HCV genotype[69]. The companies were able to produce a 500 Kg batch of the Boceprevir intermediate with a 66% yield and >99% ee. The production of the intermediate with a high enatio-selectivity and yield resulted in reduction in the cost of the process. The authors have successfully established a chemo-enzymatic route for the production of Boceprevir intermediate, where the key enzymatic reaction is the oxidation of a cyclic amine by MAO-N (Scheme 2-6). Several developments have been achieved by the authors. Firstly, the enzyme has been evolved by directed evolution to improve the activity and the stability of the biocatalyst, thereby decreasing the cost contribution of the biocatalyst. Additionally, the process limitations such as oxygen supply (through head space aeration under pressure), substrate (via feeding) and product inhibition have been addressed. The report also contains an elegant *in situ* product removal (ISPR) technique involving the degradation of the imine to the sulfonate form using bisulphite to remove the inhibitory compound. It should be noted that since the bisulfite reaction also uses oxygen, the use of ISPR results in a higher oxygen demand in this process. Additionally, it might be necessary to have a stoichiometric excess of the bisulfite to account for the competing reactions. The excess of bisulfite used could contribute to a

decrease in the efficiency of ISPR technique which increased oxygen demand adds to the cost of the process. This is the first example of a process that has been established at industrial scale using MAO-N. A process flow-sheet for the industrial process is depicted in Figure 2-2.



Scheme 2-6: Production of Boceprevir intermediate (Drawn based on process described in Li et. al., 2012).

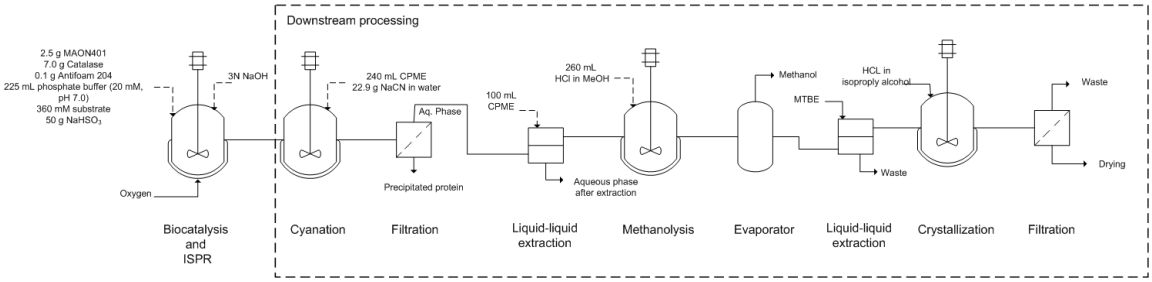


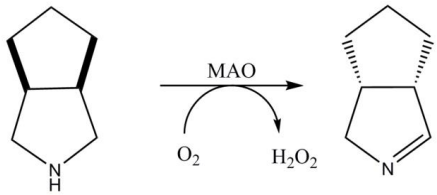
Figure 2-2: Production of Boceprevir intermediate. The process flow-sheet was drawn based on the description in the supplementary material from Li and co-workers[69].

2.5 Motivation for case study 1

Köhler et.al. first indicated that MAO can be used for oxidation of pyrollidines, and subsequently the potential to produce the drug intermediate for treatment of Hepatitis C was identified[57]. Telaprevir has been shown to have a more sustained response compared to Boceprevir for treatment, making this example particularly interesting[70]. Chemical synthesis of Telaprevir consists of lengthy steps which involves protection and deprotection of functional groups[71]. Following this, a new and improved synthesis method was suggested where the authors reduced the number of steps required for producing Telaprevir[68]. The imine formed by the biocatalytic oxidation of a cyclic secondary amine could then be used in a 3 component chemical synthesis to produce the drug molecule, Telaprevir, which is also used for the

treatment of Hepatitis C[68]. In this thesis, the imine intermediate was produced by oxidation of amine to reduce the number of steps involved in the production of the molecule. From a process stand point, use of biocatalysis to produce the imine product reduces the number of steps involved in the production of the final molecule, consequently reducing the process cost for the drug molecule. Scheme 2-7 represents the biocatalytic route for production of the Telaprevir intermediate.

The past studies that involve this particular system have only been a proof of concept and the system has not been quantified. Therefore, it is of interest to evaluate this system by identifying the limitations to the system for process development and scale-up. The Boceprevir process cannot be used directly for the process development for this system because of the potential differences in the interaction of the substrate and product with the biocatalyst as well as the differences in the physiochemical properties of the reaction species. This target reaction will be discussed further in Chapters 5 through 9.



Scheme 2-7: Production of Telaprevir intermediate using MAO-N.

In order to evaluate the reaction system for industrial implementation, the status of the reaction in terms of economic metrics (product concentration and biocatalyst yield in particular) were assessed. Also, improvement of the reaction system on the economic metrics by alleviating the process limitations was assessed.

3. Oxygen sensors as supporting tools for oxidase based biocatalysis

Oxygen plays a central role as an electron acceptor in oxidase-based biocatalysis. Oxygen is required in stoichiometric quantities for a reaction. It is therefore advantageous to measure oxygen concentrations in the reaction system. Some oxidase-based reactions operate in a two-liquid phase system that contains a solvent to aid in substrate solubilisation. In such cases, use of conventional oxygen electrodes become complicated because solvent can cause the membrane (often made of silicone polymers) to swell, resulting in measurement errors. Advanced solvent-resistant sensors have been developed recently which could be useful for such applications.

This chapter gives a short review on the implementation of monitoring tools and introduces the uses of advanced oxygen sensors.

3.1 Introduction

As mentioned in a previous chapter, oxidase-based biocatalytic processes are gaining importance and are increasingly being implemented at industrial scale. In order to develop a process for an oxidase-based reaction, it is often desired to collect kinetic data for the biocatalytic system (similar to the ones that will be discussed in Chapter 7). However, conventional methods of analysis are prone to errors. Firstly, there exists an error introduced by manual handling of the components and secondly a systemic method arising from analytical instrument. Of these errors, the systemic error cannot be completely avoided. Therefore lots of efforts have been focused on developing automated, high-throughput systems for data collection in biocatalytic systems[72,73]. Consequently, the need for sensors and other high-throughput detection methods which can be employed for online monitoring and control for use in systems has increased[74]. Several online measurement devices are available for data collection including NIR, FT-IR and LC/MS[75,76].

One field within bioprocessing where these sensors are being employed successfully for monitoring and control is fermentation. A list of sensors used for fermentation is given in a paper[77]. Conventional oxygen and pH probes have been successfully employed for several years. It is also possible to use flow cytometry in combination with fermentation to obtain online monitoring of cell structure (a recent publication has shown the application of flow cytometry for identification of yeast budding states[78]).

Biocatalysis is another field which could benefit greatly from online sensors and measurements. Oxidation reactions in particular offer an interesting possibility owing to the consumption of oxygen during the course of the reaction. Coupling an oxidation reaction with a measurement device (oxygen probe), consumption of oxygen can be traced and potentially be used as a measure for the degree of reaction. This type of application is similar to using an oxygen electrode in fermentation for measuring dissolved oxygen concentration and controlling the feeding rate of the substrate.

3.1.1 Online sensors for oxygen measurements

Oxygen sensors are very useful for fermentation processes and other applications where monitoring of dissolved oxygen is necessary. Conventionally, oxygen measurements are carried out by amperometric Clark-type electrodes[79]. The principle of measurement is electrochemical in nature[80]. The construction and working principle of such electrodes have been described in a review[81]. In such sensors, often the limiting step is the transfer of oxygen from the bulk solution through the membrane[81]. This would cause increased response times of such sensors. Also, these electrodes are required to be polarised by applying a certain voltage for a few hours before they can be used. These limitations have led to effort being placed on more robust sensors.

Modern sensors are optical sensors which consist of an oxygen-sensitive membrane, a light source (laser or light emitting diode), optical fibres and a detector[82,83]. Micro-sensors with similar principle of operation have now been developed for use in high-throughput systems[110]. Development of such versatile oxygen sensors have led to interesting studies with biocatalytic systems[84-86]. All these studies have looked into interesting aspects of oxygen transfer and application of the sensors have led to increased process understanding.

3.1.2 Applications for advanced oxygen sensors

Three important, recent applications which have led to improved process understanding (including mass transfer in microreactors, through immobilized beads etc.) are discussed in this section.

Oxygen transfer in micro-channels

Oxygen transfer in micro-channels determines the kinetics of oxygen-requiring reactions occurring in these reactors. It is therefore desirable to measure the oxygen concentrations at different positions within a microchannel. Measurements of oxygen in micro-channels have been studied by phosphorescence and fluorescence. These studies indicated measuring oxygen concentrations in microfluidic channels might be valuable[87,88].

In another study employing microreactor, oxygen concentrations were measured by having luminescent nanoparticles (245 nm) in the influent aqueous stream, using a LED to trigger the excitation and camera to image the luminescence on-line. Glucose oxidase (GOx) was used as a model system. This study compared experimental results to modelling and showed that in a typical Y-shaped microchannel, steady state can be accurately described by disregarding diffusion by convection[86].

Measurement of intra-particle oxygen gradient in solid supports used for immobilization

Immobilization of oxidation biocatalysts are often limited by the low specific activity of the immobilized biocatalyst when compared to free enzyme. The low specific activity observed is often attributed to the lack of availability of oxygen within the immobilized particles. Oxygen sensors have been used to identify the concentration gradient from the bulk solution and the internal environment. In a particular study, the authors used a methacrylic polymer (which is often used for immobilization). These beads were labelled with ruthenium complex (oxygen sensitive dye) and the biocatalyst was immobilized onto the particles. Phase shift between the excitation and the luminescence in the presence of oxygen was used to study the oxygen concentrations within the particle. GOx was used as the model system. The study indicated that the drop in oxygen concentration was related to the biocatalyst concentration. The authors indicate that such measurements could be valuable for selection of resins for immobilization as they affect oxygen concentration available by affecting the diffusivity[84].

This study was extended to an amino acid oxidase system where the authors have identified that pore diffusion (in the immobilized particles) is responsible for mass transfer resistance. In the same study, the authors have successfully established *in situ* measurements of oxygen concentration to measure the success of immobilization technique. Measurement of specific activity of the biocatalyst immobilized in two different modes (covalent and non-covalent immobilization) of immobilization indicated that covalent immobilization led to more loss in activity of the biocatalyst[89].

Measurement of oxygen transfer rates in a solvent system

Advanced oxygen sensors have been applied in high-throughput systems or in systems where process understanding is desired (two examples have been discussed in the previous section). Oxygen plays a central role in oxidase-based biocatalytic process as an electron acceptor. The reaction rates are affected by the availability of oxygen. However, solubility of oxygen in aqueous phase is low (7.95 mg/L in water at 30 ^{o}C[90]). Therefore, is desirable to improve the availability of oxygen in the system. One of the suggestions in literature for improved oxygen supply is to couple aeration with an oxygen-vector (commonly a solvent) which is capable of solubilizing more oxygen than water (this method has been adopted for improving

oxygen transfer in fermentations). By using a solvent, the oxygen availability in the reactor is improved and consequently aids as an oxygen supply strategy. However, the oxygen transfer rates reported are usually coupled to the overall mass transfer in the system rather than mass transfer from the solvent phase to the aqueous phase. A publication by McLellen and co-workers have included the difference in solubility of oxygen in aqueous and organic phases and reported the transfer rates[91]. However, this was done for a two-phase, well-mixed aerated system. Decoupling of oxygen transfer rates from air to solvent phase and subsequently from solvent to aqueous phase could provide a valuable insight for process understanding of the limiting step for oxygen transport. Also, by doing this, it is possible to assess the feasibility of using a solvent for enhanced oxygen supply. Separation of the transfer rates of oxygen from air to solvent and solvent to aqueous phases can be achieved by using a Lewis cell where the aqueous and organic phases are separated. However, conventional Clark type electrode contains a membrane made of polymers and they are seldom stable with exposure to pure solvents. Development of luminescence-based oxygen sensors (by PyroScience) has opened up the opportunity of sensing oxygen concentrations in pure solvents as well as in two-phased systems.

Some work done in this thesis (Chapter 4) has exemplified the use of solvent-resistant oxygen sensor for the measurement of K_La (oxygen mass transfer rates) in a solvent phase of a Lewis cell. These experiments goes to validate the use of the sensor for measuring the oxygen transfer rates in pure solvent which can be highly advantageous as it helps to understand the process. One could in extension also use the sensors for measuring the reaction rates or as a tool for solvent selection for two phase reactions as one could potentially track the reaction progress with the sensors.

Part II

4. Measurement of oxygen transfer in organic solvents using optical sensors

The previous chapter provided a brief overview of the applicability and uses of oxygen sensors and introduced novel oxygen sensors.

This chapter describes the use of advanced-oxygen sensors developed which are capable of working in the presence of organic solvents. Conventional Clark type electrodes used for oxygen measurements are not suitable for use in the presence of pure organic solvents. This is because the membrane in these sensors is prone to swelling in the presence of organic solvents and consequently lead to errors in measurement. Consequently, development of sensors that can be used in the presence of organic solvents present an important advance in the development for oxygen sensors. To demonstrate the applicability of such solvent-resistant oxygen sensors, separate experiments were conducted in a Lewis-cell (where the sensors were used in the presence of diisononyl phthalate-water and heptane-water systems). The experiments describe the first efforts towards characterising the oxygen sensors and measuring oxygen concentrations in organic solvents.

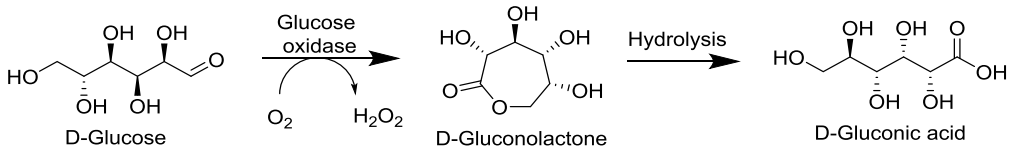
4.1 Introduction

Molecular oxygen has been established as a benign and powerful oxidant for performing synthetic chemistry[24]. However, green chemistry using oxygen suffers from selectivity issues[21,92]. Biocatalysis has provided a valuable tool to overcome the selectivity issues since enzymes are usually highly selective[23,24,93]. Consequently implementation of biocatalytic oxidations in fine chemical production has seen rapid increase[17,69,94-96]. One of the requirements for oxidation reactions is the supply of oxygen to meet the demand of the biocatalysis. Oxygen supply rate (from the gas to liquid) at scale is limited to 100 mmol/L/h when supplying with air[97]. Oxygen demand is a function of biocatalyst concentration as the rate of the reaction is affected by the catalyst concentration and the oxygen is required in stoichiometric amounts. Therefore, improvement in oxygen supply strategies may be required when high rates are required (for example, when high biocatalyst concentrations are used)[98]. Use of an auxiliary phase as an “oxygen vector” has been suggested previously especially in fermentations where the oxygen demand for the growth of the cells is critical[91,99]. This strategy for oxygen supply can therefore be extended for biocatalytic reactions requiring oxygen. Oxygen supply using solvents take advantage of the high solubility of oxygen in the solvents as opposed to the solubility in aqueous phase[100,101]. However, the amount of oxygen in the system

is limited to the saturation concentration of the oxygen in the solvent and aqueous phase. Therefore, the limitation to such a system would occur from the mass transfer rates of oxygen between the different phases (i.e., gas to liquid and liquid to liquid). It is therefore interesting to measure the transfer rates between the different phases and the effect of using a solvent phase on the extent of a biocatalytic reaction.

Conventionally, oxygen concentration is measured using Clark-type electrodes[79]. Such electrodes consist of a membrane usually made of a silicone polymer which is prone to swelling when exposed to organic solvents. Therefore, the measurements from such sensors become less reliable. Recently, PyroScience has developed solvent-resistant oxygen sensors which work based on fibre-optics. Following this development, accurate, high-throughput data collection of oxygen concentration was made possible, which is highly relevant for the purpose of this study.

In order to assess the oxygen transfer rates in solvents for a biocatalytic reaction, a model system was required. A system with a highly stable biocatalyst which has been extensively studied and is easily accessible was desired for such a purpose. It is for this reason, glucose oxidase (E.C. 1.1.3.4) was chosen as a model system. Glucose oxidase is an oxido-reductase which catalyses the conversion of glucose to gluconolactone which is spontaneously hydrolysed to gluconic acid. The reaction uses oxygen as a substrate and produces hydrogen peroxide as a by-product (Scheme 1)[102]. Glucose oxidase is a well characterised system which has been used for several applications, e.g. in the food industry and in the development of sensors for glucose detection (for clinical and biochemical purposes)[103,104].



Scheme 4-1: Conversion of glucose catalysed by glucose oxidase.

The paper discusses the use of glucose oxidase in a Lewis cell in the presence of two organic solvents for testing the effect of using a solvent phase on the biocatalytic reaction. Furthermore, oxygen transfer coefficient (Kla) measurements were conducted. Finally, other applications for solvent resistant sensors have been suggested.

4.2. Materials and methods

Potassium di- and mono-hydrogen phosphate and glucose were purchased in technical grade from Sigma Aldrich (Steinhiem, Germany) and used as purchased. Solvents used in the study were purchased in analytical grade, also from Sigma Aldrich. GOx was kindly donated by Novozymes A/S (Bagsværd, Denmark). The solvent-resistant oxygen sensors were procured from PyroScience (Aachen, Germany) and the FirestingO2 was purchased from PyroScience. The software for data collection (Oxygen logger v.3.1) was downloaded from PyroScience website (http://www.pyro-science.com/downloads.html).

4.2.1 Reactors

The experiments were carried out in a 250 mL stirred tank reactor equipped with two Rushton turbines (6 blades, diameter 2.4 cm, width of the baffle was 0.6 cm and height 0.5 cm) and two baffles (of 1 cm width). The height of the impeller and the stirring speeds are adjustable.

4.2.2 Calibration of oxygen sensor

A 2-point calibration was made for the oxygen sensors by exposing them to air (for 100% DO) and 1% sodium sulphite (for 0% DO).

4.2.3 Kla measurements

Kla measurements were made using dynamic gassing out technique[105] using nitrogen for gassing out and head space aeration as a means of oxygen supply.

Kla measurement for aqueous phase

To a stirred tank reactor, 40 mL of 200 mM phosphate buffer (at pH 7.0) and 10 mL of glucose (at 50 g/L) was dispensed. The reactor was stirred at a speed of 72 rpm at 37 ^{o}C. The buffer was sparged with nitrogen until the oxygen concentration stabilizes at 0. Following this, nitrogen was stopped and the reactor the oxygen transfer measured using the sensors. Two sensors were used at opposite either sides of the reactor (close to the baffles).

Kla measurement for organic phase

50 mL of heptane was taken in the stirred tank reactor (stirred at 72 rpm at 37 ^{o}C) and the head space aerated with nitrogen until the oxygen concentration in the aqueous phase stabilized at 0. Following this, nitrogen gas flow was stopped and the reactor left stirring to equilibrate with oxygen in the head space and the increase in oxygen concentration documented with two oxygen sensors (one attached to each baffle).

Kla measurement for two phase system

To simulate a two phase Lewis cell system, 40 mL of phosphate buffer, 10 mL of 50 g/L glucose and 50 mL of heptane were taken in a reactor which was maintained at 37 ^{o}C at 72 rpm. Stirring was maintained such that the aqueous and organic phases existed as two separate layers. Nitrogen was supplied through head-space aeration until the oxygen concentration in both the aqueous and organic phase stabilized at zero. Following this, the flow of nitrogen was stopped and the increase in oxygen concentration measured with one sensor in each phase. The experiment was repeated to get a duplicate measurement.

4.2.4 Biocatalysis

Glucose oxidase (GOx) based biocatalysis was carried out to convert glucose to gluconic acid. The oxygen concentration was measured using the solvent-resistant oxygen probes. The reaction was carried out in 250 mL stirred tank reactors at 37 ^{o}C. Biocatalysis in such reactors were operated in both single and two-phase systems.

Substrate inhibition

To the reactor different volumes of 50 g/L glucose stock was dispensed with buffer (200 mM, pH 7.0) such that the concentration of glucose was 2, 4, 8, 12, 24 g/L. 1 mL of 0.1 g/L GOx was added to get a final GOx concentration of 0.01 g/L. The rate of oxygen consumption was measured with two oxygen sensors, each placed at one baffle.

Biocatalysis in Lewis-cell

All biocatalysis in Lewis cell was carried out at a substrate concentration of 10 g/L. The working volume of the aqueous phase was 50 mL and the pH was maintained at 7.0 by 200 mM phosphate buffer. The reactor was maintained at 37 ^{o}C and 72 rpm. To the aqueous phase, 50 mL of the organic phase (depending on the experiment, it was heptane or diisononyl phthalate) was added. Two impellers were used for stirring (one in the aqueous phase and one in the organic phase). When the oxygen concentration measurements were stabilized, biocatalyst at a concentration of 0.01 g/L was added to the aqueous phase through a feed pipe. The oxygen depletion in each phase was measured with the oxygen sensor.

4.3 Results and discussion

Use of oxygen sensors has only been exploited for measuring the oxygen concentration in the reaction system. However, it can be used for several other applications that involve characterising oxygen requiring reactions. In this paper, oxygen sensors that are resistant to solvents were used to demonstrate their

applicability as tools for oxidase-based biocatalysis. One of the scopes of this study was to test the applicability of the oxygen sensors for measuring initial rates of the reaction system. Secondly, the effect of adding a second phase on the oxygen transfer in the system has been studied.

4.3.1 Substrate inhibition

Substrate inhibition for biocatalytic reactions is a common occurrence. In order to assess substrate inhibition, the initial reaction rate at varying substrate concentration is measured (Initial rate was calculated over the first 30 s after addition of the enzyme). The sensor that has been used in this particular study can record the oxygen concentration every second making the analysis more accurate. The consumption of oxygen is measured as partial pressure from the oxygen sensor. Partial pressure can then be converted to concentration using Henry's Law. Henry's constant for oxygen in aqueous phase is assumed to be 1.16. The study Figure 4-1 indicates that inhibition of the biocatalyst by the substrate is evident at concentrations of 80 mmol/L and higher. The results are in line with substrate inhibition that has been reported previously[106].

Conventional methods often incorporate the use of standard analytical equipment for measuring the substrate and product profile over a period of time. The downside of adopting this technique is that it is time consuming and often includes errors arising from sampling (manual handling of the samples). One could reduce the analysis time by implementing online oxygen sensors while also improving data quality by sampling more often. Therefore, use of such sensors for initial rate measurements can prove to be a useful tool for fast data collection.

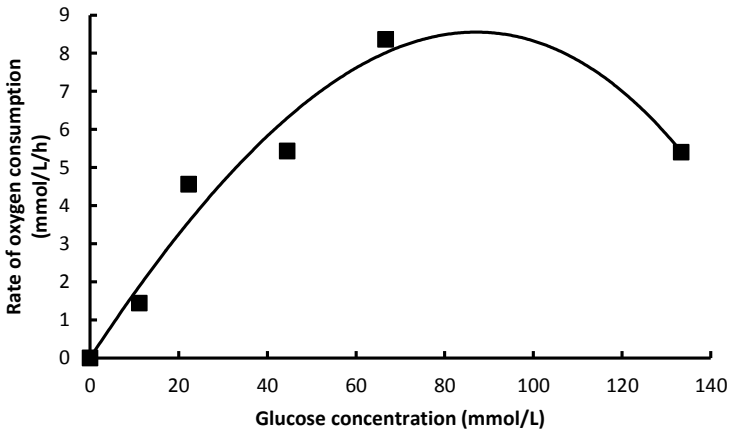


Figure 4-1: Substrate (glucose) inhibition profile for a glucose oxidase system.

Substrate inhibition curve obtained has been used to obtain the optimum substrate concentration for the reactions with a two-phase system. For the following studies with a two phase system, substrate concentration where the reaction rate is not limited by substrate inhibition was chosen in order to maintain the highest possible reaction rate in the aqueous phase. Therefore, a concentration of 60 mmol/L of glucose was used.

4.3.2 Solvents as vectors for oxygen transfer

In order to test for the oxygen supply through the use of solvents, two solvents whose LogP > 4 and were in the list of environmentally safe solvents from the GCI solvent selection guide were chosen[107]. Solvents with Log P greater than 4 were chosen because they are less toxic to the biocatalyst. The solvents chosen for the test were heptane and diisononyl phthalate.

Biocatalysis in a Lewis-cell

A modified Lewis cell[108] where two liquid phases were present such that the aqueous phase was at the bottom and the organic phase was at the top was used in the experiments conducted in this study (Figure 4-2). Lewis cells have been previously used in two-liquid phase reactions where the solvent phase was used as a substrate reservoir and/or remove product from the aqueous phase[109,110]. Mixing in each phase was achieved by use of a Rushton turbine mounted on a shaft propelled by an overhead motor. Since the two stirrers were mounted on the same shaft, the stirring speeds in the aqueous and organic phases were kept a constant and the Rushton turbine was placed such that it was placed in the middle of each phase. A Lewis-cell was used instead of a well-mixed system so that the surface area available for oxygen transfer between the organic and the aqueous phase could be maintained a constant (and could be calculated) irrespective of the solvent used.

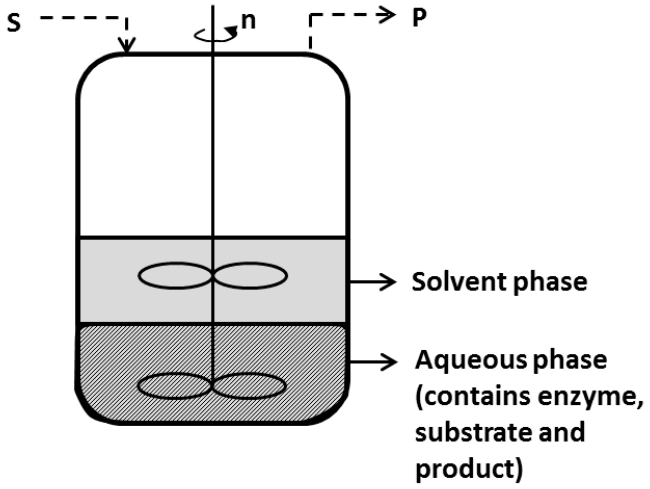


Figure 4-2: Depicts a Lewis cell with two liquid phases. In this particular example, the substrate and product are highly soluble in aqueous system and the oxygen transfer occurs from the solvent phase to the aqueous phase through the interface present.

In this particular example, the conversion of substrate to product occurs at the aqueous phase. The solvent phase acts as a reservoir for oxygen and oxygen transfer occurs from the headspace to the solvent phase and subsequently from the solvent phase to the aqueous phase. The oxygen concentrations were measured at the aqueous phase and the organic phase simultaneously and recorded to give an idea of the reaction rates. Oxygen concentrations were measured as partial pressure and depicted in Figure 4-3. The figure indicates that the oxygen concentration is almost constant in the organic phase whilst there is a rapid reduction in the oxygen concentration in the aqueous phase.

From the rate of oxygen consumption, the rate of the reaction can be calculated. Reaction rates were also calculated in the absence of solvent and compared to the reaction rates obtained in the presence of an organic solvent. The relative rates obtained in a Lewis cell was compared to that of an aqueous phase and reported in Table 4-1.

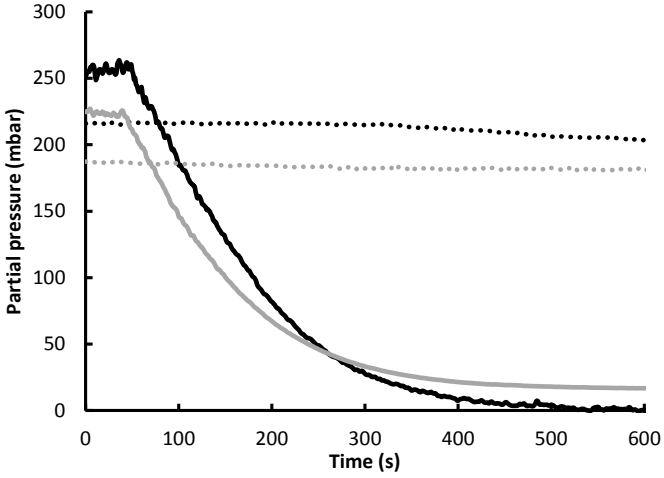


Figure 4-3: Partial pressure of oxygen in a Lewis cell-system using an aqueous phase together with an organic phase of either diisononyl phthalate (represented by black line) and heptane (represented by grey line). The dotted lines represent the oxygen concentration in the organic phase and the solid lines represent the concentration in aqueous phase.

Table 4-1: Relative rates obtained for reactions run with an organic phase to the rate obtained from a reaction without solvents.

Solvent	Relative activity
None	1
Heptane 1	1.10
Heptane 2	1.02
Diisononyl phthalate1	1.02
Diisononyl phthalate2	0.93

From Table 4-1, it can be seen that the presence of a solvent phase does not cause an increase in the reaction rate. It has also been observed that the oxygen concentration in the solvent phase is almost a constant through the reaction. This indicates that the rates of oxygen transfer could be a limiting factor in the reaction. To validate this theory, the oxygen transfer rates from the headspace to the organic phase and the organic phase to the aqueous phase was measured by dynamic method. Nitrogen was flushed through the aqueous phase, and when an organic phase was present, the oxygen concentration was depleted by sweeping the headspace with nitrogen. This was done to decrease evaporation of the organic phase. The oxygen saturation phase was obtained through headspace aeration to mimic the transfer rates in the Lewis cell when a biocatalytic reaction is carried out. In principle, when the nitrogen sweeps out the oxygen from the system, the oxygen concentration becomes zero. When headspace aeration is then started, oxygen concentration rises in the system until it reaches saturation (as seen in Figure 4-4). It is

important that the sensors used for Kla measurements have a low response time in order to avoid the errors involved in measurement[111].

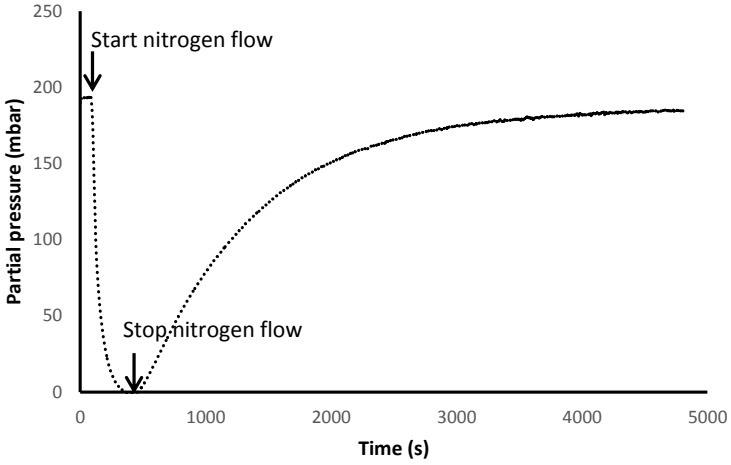


Figure 4-4: Dynamic method for determination of oxygen transfer rates from the headspace to heptane system.

From the results obtained in Figure 4-4, the oxygen transfer rate (Kla) can be calculated for heptane. Kla represents the mass transfer of oxygen where Kl represents the rate constant and a represents the surface area per unit volume. Kla was calculated from the partial pressures obtained by the oxygen sensor since the concentration is related to the partial pressure by Henry's law. Therefore, Kla can be calculated using the following expression:

$$\frac{dP}{dt} = P_{sat} - P^{*}_{sat} * e^{-Kla*t}$$

Where dP/dt represents the rate of change of partial pressure, Psat is the partial pressure, Psat* represents the partial pressure at saturation concentration of oxygen, Kla represents the mass transfer coefficient and t represents the time.

Duplicates were obtained by using two oxygen sensors within the reactor at the same position on the baffles. After the Kla were calculated, the values were used to simulate the data points to validate the fit for the duplicates. This showed a good correlation to the experimental result (results not shown).

Furthermore, since the area available for oxygen transfer in the Lewis cell is fixed by the diameter of the reactor (6.5 cm) and the total volume of the aqueous phase (50 mL), Kl values can be calculated from the

measured Kla. Table 4-2 gives the Kla values and calculated Kl values for transfer of oxygen from the headspace in a single phase and two phase system. Oxygen transfer from air to aqueous phase was measured for comparison and reported in the table. The Kl values reported for a two phase system in a similar Lewis cell experiments by Woodley and co-workers is in the same order of magnitude as the ones obtained in this study[109].

Table 4-2: Kla and Kl values obtained for single- and two-phase systems.

Particulars	Kla (h^{-1})	Kl (mh^{-1})
Air →buffer containing glucose	7.2	$5.4*10^{-2}$
Air → heptane	4	$3.0*10^{-2}$
heptane →buffer containing glucose	3.5	$2.6*10^{-2}$

Based on the Kla values reported in Table 4-2, it can be seen that the limiting transfer rate is between the liquid phases. It should be noted that the oxygen transfer from the organic phase to the aqueous phase is the limiting factor.

The factor limiting the improvement in reaction rate by use of a solvent in this case can therefore be concluded to be the transfer of oxygen from the organic phase to the aqueous phase. This can also be seen in Figure 4-3, where the oxygen concentrations in the organic phase hardly decrease. Therefore, it might be interesting to test the oxygen transfer rates in a well-mixed system where the organic phase is dispersed through the aqueous phase.

4.3 Applications for oxygen sensor in oxidase-based processes

This paper has indicated the use of advanced-oxygen sensors for rapid measurement of oxygen transfer rates in a solvent-based system. The sensors are particularly interesting for Kla measurements because they exhibit high stability in high vapour pressure organic solvents, have a low response time and can record the oxygen concentration as frequently as every second. Reduction in the response time as well as the increase in the frequency of recording improves the quality of the measurements making it possible to obtain more accurate oxygen transfer rates.

Also, the developed oxygen sensor was applied to the characterisation biocatalyst inhibition profile through the measurement of initial rates. It is noteworthy that care should be taken when adopting this method of analysis for quantitative assessments. This is because several reaction characteristics affect the oxygen concentration. For example, most glucose oxidase preparation contains catalase. If catalase is present, the hydrogen peroxide produced in the system is broken down to water and oxygen. Therefore, the oxygen

concentration measured will be a net effect of the oxygen consumed by the reaction and the oxygen produced from the degradation of hydrogen peroxide. The presence of catalase and the rates of degradation of hydrogen peroxide relative to the reaction should be characterised through separate experiments and accounted for.

Initial rate measurements can also be used for screening purposes. For example, the oxygen sensors can be used for screening oxidases from different sources for catalysing a select reaction. Also, solvents for applicability in two phase systems could be screened by measuring the oxygen consumption during the reaction.

Since stoichiometric amount of oxygen is consumed by an oxidase system (non-whole cell based processes which do not contain catalase), the rate of oxygen consumption can be used for feeding a substrate to the reaction system. As an extension, the sensors can be used for online monitoring of the reaction progress. However, this needs to be done in combination with standard analytical methods to obtain quantitative information on the reaction yields. For instance, the oxygen sensors can be used to give an idea about whether the reaction has reached completion or not. However, the reaction yield can only be obtained when the substrate and product concentrations are analysed. Application of such sensors becomes particularly interesting in continuous oxidations. Oxidation reactions at steady-state would contain a said oxygen concentration. An increase in oxygen concentration would be observed when the catalyst loses its stability. Therefore, stability of a biocatalyst can be assessed on-line.

4.4 Conclusions

In this chapter, advanced oxygen sensors were used for measuring volumetric mass transfer coefficients in an organic phase. The study indicated that these sensors can be used for quick and high-throughput data collection. The application of such sensors in a two-phase system in a Lewis-cell has been used as a case study to investigate the several options for the use of the solvent-resistant sensors. The results indicated that operating an oxidase reaction that is as fast as a glucose oxidase in a Lewis cell is not advantageous because liquid-liquid mass transfer is the limiting. Finally, other potential uses for the sensors have been identified. In the following chapters, a different oxidase based biocatalytic process has been discussed. The oxygen sensor used in this investigation can potentially be used in the following case study.

Part III

5. *In silico* Analysis of Potential Limitations

To successfully scale-up of a process, its characteristics and limitations (if any) need to be identified. This knowledge is the basis for a pragmatic methodology to overcome said limitations and also suggest where the efforts for improvements need to be placed (for example, biocatalyst development through protein engineering, process improvement through ISPR etc.). Constraints to the system can be broadly classified as process-related (such as change in pH, oxygen supply etc.), reaction-related (pertaining to the physicochemical properties of the substrate(s) and product(s)) and biocatalyst-related (inhibition, stability of the enzyme, specific activity etc.). Often, knowledge of the process is gained through experimentation that requires a large investment of time and effort. However, some process limitations can be identified by taking a closer look at the target reaction system. By analysing the physicochemical properties and the targets required for a given reaction, it is often possible to identify which experiments have a higher priority, reducing the load on the time and effort placed on process development.

In the Part I, the motivation behind selecting a monoamine oxidase based syntheses was presented. This chapter describes the efforts towards identifying potential bottlenecks for the MAO case study prior to experimentation. By doing this exercise, the experimental load can be focussed on the expected limitations and help in arriving at smart solutions.

5.1 Introduction

Biocatalytic reactions are becoming increasingly popular in industrial processes because of the selectivity and the diverse chemistry they can offer. However, biocatalysts are often operated in conditions that differ from their natural environment[112]. Based on the reaction characteristics and targets, several process options (which include choice of reactor, mode of operation, development of biocatalyst among other tools employed in the process such as in situ product removal, substrate feeding strategy etc.) can be used to achieve the set goal (eg. product concentration, reaction time etc.) for a given process. The choice of a particular process option (for the given process) would depend on the knowledge of the reaction and/or the constraints (time and financial) faced by the industry. However, complete knowledge of the reaction system is seldom available due to the lack of integration of product and process discoveries and experimentation is required to gather relevant data. In order to reduce the time and investment spent on experimental development of the process, it would be useful to make some pragmatic choices on the

number of process options that are worth investigating for a particular reaction, based on its characteristics. It is with this purpose in mind that an *in silico* analysis of potential process limitations was carried out. The analysis serves to direct experiments to select conditions and help reduce experimental load.

5.2.1 Process limitations

A simplistic view of a biocatalytic reaction can be explained by the binding of the substrate to the active site of the biocatalyst (forming an enzyme-substrate complex), conversion of the substrate to the product (formation of enzyme-product complex) and release of the product from the active site. Over the course of the three steps several constraints can arise.

First of these is the availability of the substrate for binding with the biocatalyst. Factors that affect the availability of the substrate are dictated by the substrate properties such as its solubility, volatility, melting and boiling point among others. In cases where an immobilized biocatalyst or a whole-cell biocatalyst is used, it is likely that the availability of substrate is further limited by its mass transfer.

Another constraint arises from the binding of the substrate to the active site of the biocatalyst. For example, the substrate can irreversibly inactivate the biocatalyst (when the substrate binds irreversibly to the biocatalyst such that it prevents the formation of product), a phenomenon referred to as substrate inactivation.

A further constraint that can arise is attributed to the dissociation of the product molecule from the active site. Herein, the uncoupling rate dictates the rate of product formation. If the product binds irreversibly to the biocatalyst or causes modifications in the biocatalyst, it is said to be toxic. If the product molecule produced binds to the enzyme such that it prevents binding of new substrate molecule to the active site, it is said to be inhibitory.

Other characteristics of the product can also cause product loss or loss of biocatalyst stability. For example the reaction could be endo- or exo-thermic causing the reaction to slow down or the biocatalyst to lose its stability unless the temperature is controlled. The product could be volatile causing product loss.

Finally, limitations can also arise that are related to the reactor or process which include supply of oxygen, control of pH etc.

In a broad perspective, the limitations to a reaction system can be classified into biocatalyst-related limitations (eg. inhibition, toxicity, stability), reaction-related (affected by physiochemical properties of the substrate and product) and reactor-related (such as need for oxygen supply or pH control). Figure 5-1 depicts some examples of reaction constraints that cause potential limitations to implementing a target process.

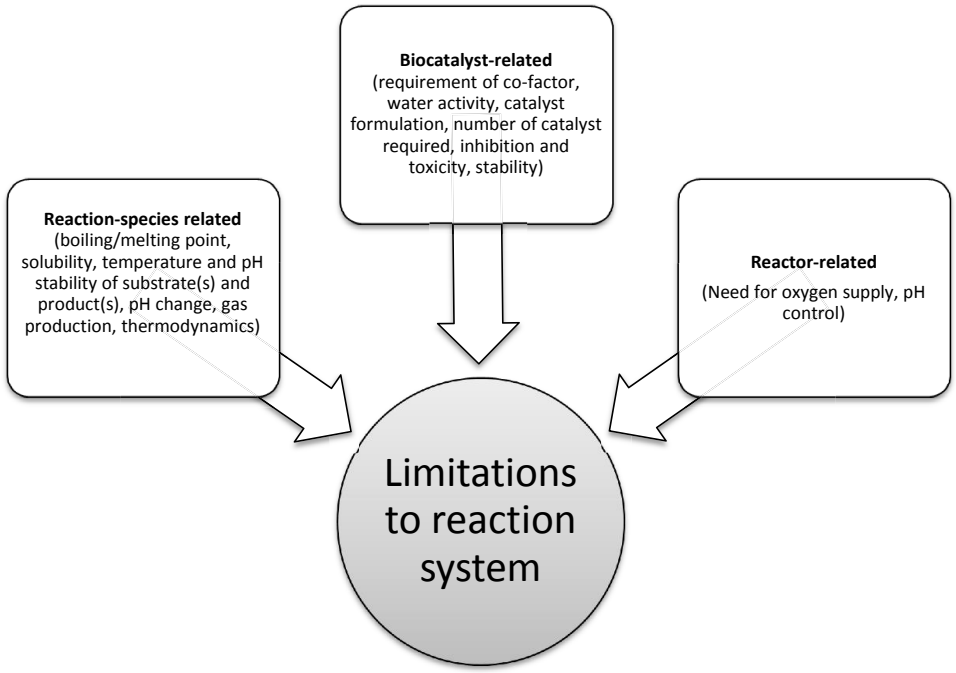


Figure 5-1: Factors contributing to limitations of reaction system. Examples of limitations are listed in each category.

In the following sections, *in silico* analyses of the potential limitations for the target system and the corresponding solutions are discussed.

5.3 Biocatalyst-related limitations

Biocatalyst-related limitations arise either from factors inherent to the biocatalyst (such as stability, requirement of co-factors and catalyst formulation) or from interaction of the reaction species with the biocatalyst. In the following section, analysis of biocatalyst-related limitations for the target system will be discussed. These limitations can arise from non-specific effects and/or specific effects in terms of inhibition and toxicity caused by the substrates (amine and the oxygen) and the products (imine and hydrogen

peroxide). These limitations will be discussed based on the literature available and is extended to the specific case study whenever possible.

5.3.1 Non-specific inactivation of the biocatalyst

For successful implementation of biocatalytic reactions, it is desired that high concentrations are produced to maintain high process intensity. Consequently, the biocatalysts used for synthetic applications are often exposed to high concentrations of substrates and products as opposed to the mild conditions that the biocatalyst would be exposed to in nature. As a result, often they are susceptible to inhibition and/or toxicity[113].

Octanol-water partitioning (Log P) has been used for evaluating the toxicity of solvents to the biocatalysts. It is argued that the biocatalyst is stripped of water by solvents with low Log P values (typically below 2). For a whole-cell system, however, the toxicity arises from the integrity of the cell membrane being lost[114]. A study of biocatalysis with whole-cells and immobilized whole-cells indicated that solvents were toxic when their Log P was over 4[115]. Straathof reported a methodology for predicting such toxicity based on the solubility and logP values of the reactants and products.

The assumptions that go with the estimation developed by Straathof include that the reaction is not limited by thermodynamics, it is not mass transfer limited and phase toxicity is neglected. After extensive study, they have shown that this estimation is valid only when the solubility of the product in water is between 0.3 and 200 mM. The concentration which causes the biocatalyst to lose most or all of its activity can be calculated using the following equations[116]:

$$logs_i^{aq} = 0.5 - 0.01(f_i - 25) - logP_i$$

$$logc_{crit}^{aq} = 0.79logs_{aq} - 0.74$$

where s_i^{aq} is aq solubility, f_i =25 for a liquid at room temperature otherwise is the melting point in ˚C.

For the target reaction, the assumptions of the methodology rule out its applicability for one of the substrates (oxygen) and the product (imine). This is because oxygen offers a second phase which could lead to potential phase toxicity and the imine has a low aqueous solubility. The methodology is not tested for hydrogen peroxide because of the potential interference by catalase present in whole-cell (it has been shown that intracellular specific catalase activities for degradation of hydrogen peroxide are relatively high[117]). However, the methodology can potentially be used to estimate the concentrations at which the

substrate (amine) reaches critical concentrations. The solubility and logP values of the substrate can be estimated by EPA's online software (EPI Suite, http://www.epa.gov/opptintr/exposure/pubs/episuite.htm), which makes the solubility prediction. Many such types of software for property prediction are available and can also be used[118]. By applying the values to the equations, the critical concentration of the amine was estimated to be 100 mM. For an economically scalable process, the processing intensity has to be high and consequently the biocatalyst is exposed to high concentrations of substrate and therefore there is a high likelihood that the substrate for the target system at these concentrations are affected by the toxicity.

Inactivation of the biocatalyst by the product has not been analysed by the above mentioned methodology, but can be expected since a similar imine was found to be toxic to a monoamine oxidase[69].

5.3.2 Specific interaction of the substrate and product with the biocatalyst

While substrate and product inhibition has not been assessed in silico, it can be expected since the biocatalyst will be exposed to high concentrations. Inhibition of a monoamine oxidase by substrate and product has been seen in the Boceprevir example[69].

Inhibition of the substrate and the product can be overcome by improving the biocatalyst (at industrially relevant conditions)[119,120] and/or employing process development tools such as *in situ* product removal (ISPR)[112,113,121,122] (in cases where product inhibition/toxicity is present) and substrate feeding or *in situ* substrate supply (ISSS)[123-125] (in cases where substrate inhibition or toxicity is prevalent). In some cases, it is desired that the substrate supply and product removal are carried out simultaneously. Use of auxiliary phases (solvents, resins) will provide an opportunity for this kind of supply[126,127].

Overcoming substrate and product inactivation

Available Substrate feeding strategies are discussed in detail in a review[125]. It should be noted that feeding of substrate should be in such a way that it matches the rate of the reaction in order to prevent the reaction from operating at substrate limited regimes. In cases of solids, it is desired to use a co-solvent to solubilize the substrate or feed the substrate as a solid. In both these cases, the availability of the substrate is limited by the solubility (when implementing solid feeding strategy) or mass transfer across the phases (when co-solvent was used). Also, adding a co-solvent can cause loss in stability of the biocatalyst. Solid feeding strategy has been implemented in a biocatalytic process catalysed by *Mycobacterium*[128].

While substrate inhibition is easily overcome by feeding strategies, implementation of ISPR techniques is rather difficult. This is because the substrate and product molecules of a biocatalytic reaction are often very similar. It is important to consider the selectivity of the product removal technique for the product over the

reactants. It is necessary to carefully consider the limitations of the type of substrate and product removal techniques employed.

5.3.3 Inactivation (toxicity) by reactive co-product

Common phenomenon to oxidase-based biocatalysis is the formation of hydrogen peroxide as a by-product. Once the hydrogen peroxide is produced, its interaction with the biocatalyst determines the effect of hydrogen peroxide on the biocatalyst. Hydrogen peroxide is known to cause chemical modifications on proteins. For example, methionine residues in the catalyst can easily be oxidised. These oxidations could in turn lead to lowered stability of the enzyme[129]. Other residues that have been seen to be affected by exposure to hydrogen peroxide include cysteine, tyrosine, and phenyl alanine among others. Protein fragmentation had been observed by Törnvall et al. on exposure to hydrogen peroxide [130]. Therefore, removal of hydrogen peroxide from the microenvironment of the catalyst desired. It can either be done with chemical catalysts or by using catalase in the system[131]. The use of catalase is preferred as they are specific and produce benign products (oxygen and water). This potentially opens up the opportunity to use the oxygen produced as the substrate for the oxidase reaction[117].

For the monoamine oxidase used for the current study, the active site residues consist of serine, aspargine, methionine and tyrosine residues close to the active site[64]. Therefore, it is likely that hydrogen peroxide mediated inactivation can occur causing the loss in stability of biocatalyst.

5.3.4 Inactivation by oxygen

Supply of oxygen is critical for oxidase-based reactions; however, oxygen supply can potentially cause deactivation of the catalyst. Proteins are often affected by the presence of interfacial effects (such as the ones occurring in the presence of gas-liquid interface which is constantly coalescing and breaking up in the reactor)[132]. While shear effects could cause inactivation in soluble enzymes, use whole-cells can offer more protection to the enzyme and this effect can be neglected. Another way of deactivation is that the amino acid residues in the enzyme can be oxidized by the formation of reactive oxygen species[133,134]. It is therefore essential to supply oxygen in such a way that the stability of the biocatalyst is not compromised[135]. Stabilization effects by immobilization and/or protein engineering could help overcome this limitation.

5.4 Reaction species-related limitations

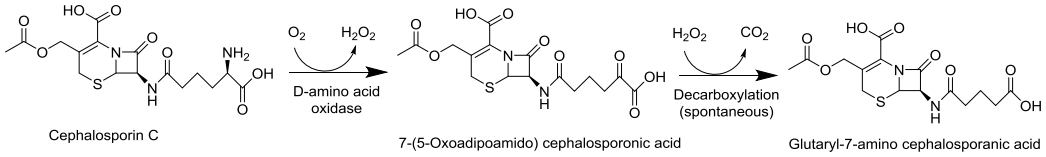
5.4.1 pH shift during the reaction

When the substrate and product for a reaction system have difference in pKa values, a change in pH during the course of the reaction is expected. Therefore, looking at the pKa values of the susbtrate and the product would give valuable insight into the pH shift that can be expected and consequently the buffer strength needed for the reaction can be assessed. The pKa values of the substrate and product can be determined from a property prediction software (EPI 4.0).

Amines are highly basic, and the corresponding imines are less basic. Therefore, a decrease in pH can be expected. For the target system, the pKa values of the substrate and product are 8.2 and 11.6 respectively. Therefore, there an acidic shift in pH can be expected as the reaction progresses. Consequently, pH control becomes critical to the process and the final product concentration that can be achieved. pH control is a process-related challenge and is discussed later.

5.4.2 Degradation of the substrate and product by hydrogen peroxide

Hydrogen peroxide could potentially cause adverse effect on the reaction by modifying the substrate or the product of the target reaction[136]. For example, in the production of 7-aminocephalosporanic acid (7-ACA) from cephalosporin C using an amino acid oxidase, the hydrogen peroxide produced during the reaction spontaneously decarboxylates the product to Gl-7-ACA. Gl-7-ACA in turn is used by another enzyme, acylase, as a preferred substrate for the production of 7-ACA (Scheme 5-1)[137].



Scheme 5-1: Spontaneous decarboxylation by hydrogen peroxide (Scheme adopted from [137])

Similarly, oxidation of the substrate (amine) by hydrogen peroxide is possible and literature was used to verify the possibility of such reactions. Although oxidation of tertiary amine by hydrogen peroxide is well known, they require high temperature (60-65 ˚C)[136]. Oxidation of secondary amine, like the substrate in the target reaction system, by hydrogen peroxide requires the presence of a catalyst[138]. Both these scenarios

are not relevant to the process conditions at which MAO reaction occurs. No such reports on oxidation of tertiary imines have been found.

5.5 Process-related limitations

5.5.1. pH control

As mentioned before, a pH shift can be expected for the target reaction system. pH shift can be overcome by using a buffer at concentration that can cope with the pH change or by coupling the reactor to a pH stat. Another approach to pH control could be to feed the substrate at a sufficient rate (below inhibition levels) and compensate for the difference with an addition of base. This would reduce the base consumption and the dilution effects on the system.

5.5.2 Oxygen supply strategies

For an oxidase based reaction, one mole of oxygen is required to convert one mole of substrate into the product. However, oxygen supply at scale is limited to 100 mmol/L/h when air is used to supply oxygen to the reactor[97,139]. Therefore, the next potential challenge for the system is development of an oxygen supply strategy to achieve the desired concentrations at a relatively economic way. Some potential means of achieving high K_La (mass transfer of oxygen) include the use of modified aeration strategies or reactors for example, the use of bubble column reactors[140,141], supplying air with a higher surface area (by reducing the bubble sizes)[142] etc. Oxygen can also be supplied at a higher rate when oxygen is used instead of air or by increasing agitation rates[143]. However, these strategies would incur additional costs. Also, care should be taken when introducing oxygen into the reactor because gas-liquid interfaces and oxygen concentration could leave to loss in stability of the biocatalyst as mentioned previously.

Since it is not possible to assess the amount of oxygen required by a target system without initial rate measurements experiments were conducted to assess oxygen requirement of the target reaction are discussed in Chapter 7.

In summary, the potential limitations of the target MAO system can be classified as biocatalyst-, reaction species- and process-related limitations and is represented in Table 5-1. In the table, potential solutions have also been listed. Each of these solutions comes with a certain limitations for implementation. Such limitations should be considered while implementing a particular solution. These comments have also been stated in this Table.

In the following chapters, experimental evaluations of the identified limitations are conducted in order to assess the presence or absence of a particular limitation.

5.6 Conclusions

Based on the literature survey and *in silico* analysis for the reaction system, several potential limitations have been identified. Through such analysis before starting the experimental work, important information about how the process should be operated can be identified. For example, by looking at the physicochemical properties of the substrates and products one can identify the limitations such as potential volatility, solubility issues. Therefore, it is a good practice to consider the properties of the reaction system prior to experimentation.

Table 5-1 summarizes the potential challenges identified by such analysis and the potential solutions to overcome these challenges for the target system. However, the extent of the limitation and the applicability of the solutions have to be assessed experimentally. The presence or absence of the limitations and the quantification of the limitations present have been identified through experimentation and will be discussed in Chapters 6-8.

Table 5-1: List of potential challenges for MAO case study and the possible solutions.

			Potential challenges identified through in silico analysis of MAO catalysed reaction								
			Biocatalyst-related					Reaction species-related		Process-related	
			Potential substrate inhibition	Presence of product inhibition	Presence of co-product inihbition	Oxidization of amino acid residues in biocatalyst	Loss of stability of biocatalyst during reaction	pH change during reaction	Presence of product volatility	Need for oxygen supply	Comments(s) on implemented solution
Potential solutions that can be implemented	Biocatalyst engineering	Modify catalyst formulation				√	√				Use of whole-cells in an oxidation process could offer greater stability to the biocatalyst. It can also cause mass tranfer limitation. Additionally, it can cause increased demand for oxygen.
		Improve biocatalyst	√	√	√		√				Biocatalyst improvement can be made such that the biocatalyst is less inhibited by modifying the affinity of the substrate and product to the enzyme. However, a threshold is present for the affinity of the substrate molecule to the biocatalyst.
		Immobilize biocatalyst				√	√				Immobilization of the biocatalyst improves the stability of the biocatalyst, but it adds to the process cost. Additionally, oxygen transfer and mass transfer might be affected.
	Reaction Engineering	Use catalase as co-catalyst			√					√	Addition of catalase as a co-catalyst to cope with hydrogen peroxide would add to the process cost. The success of the system depends on the accessibility of the hydrogen peroxide to the catalase. The catalase load should match the rate of hydrogen peroxide production.
		Use solvents	√	√						√	Solvents can be used to supply oxygen to the rection owing to their increased solubility. They can affect the stability of the biocatalyst and the availability of susbtrate. When used as an ISPR technique, selectivity of the product over substrate should be taken into account. Safety concerns arise when using solvents with oxygen supply.
	Reactor engineering	Implement bubble-column reactors								√	Bubble-column reactors can be used for improved oxygen supply. However, more bubbles might lead to lowered stability of the biocatalyst.
		Supply pure oxygen								√	Use of pure oxygen to supply oxygen at a higher rate would increase process cost and cause a potential decrease in enzyme stability.
		Implement ISPR		√			√	√	√		The success of ISPR depends on the difference in the physiochemical properties of the substrate and product.
		Feed base/use buffered systems						√			Addition of base or use of buffered system can be used to maintain pH. To prevent dilution, addition of concentrated base is desired, however, this could cause loss of biocatalyst's stability.
		Implement substrate feeding-strategies	√					√			Feeding of substrate should consider the rate of the reaction. Feeding is limited by the reactor size and the size of the reservoir.

6. Reaction Species-Related Limitations

In the previous chapter, the potential limitations to the target system arising from the reaction-species, biocatalyst and process were identified. The limitations that arise solely from the nature of the reaction species, i.e. the physiochemical properties of the substrate and the product are illustrated in this chapter.

For the monoamine oxidase case study, the first of such limitation arises from the product (imine) having a lower pKa value (pKa = 8.2) than the substrate (amine, pKa = 11.6). During the course of the reaction, as the imine is produced, there will be a pH shift. Such a pH shift will cause the reaction to either slow down (if the biocatalyst is active over a wide range of pH) or in some cases when the enzyme has a steep pH optimum, the reaction can terminate before the biocatalyst has been exploited to its full potential.

Also, Köhler and co-workers reported that the imine formed (from the target reaction) can co-exist in solution as a trimer. This would imply that the process can't be followed by the formation of product (since the trimer is not detected in the gas chromatography).

Additionally, for a similar biocatalytic system, the product imine was found to be volatile[69]. This could be the case for the target system and needs to be evaluated.

This chapter deals with analysing these product-related limitations and goes on to confirming or validating the presence of one or more of these limitations through experimentation. Finally, some tools to overcome the limitations have been suggested (which would further affect the design considerations for the process).

Product-related limitations can arise from the physicochemical properties of the product (pKa value, volatility, stability at reaction conditions) or from the interaction of the product with the biocatalyst (e.g. toxicity).In the following sections, the product-related limitations that have been assessed from literature have been presented.

6.1 Introduction

In the following sections, product-related limitations identified in the previous chapter are quantified.

6.1.1 pH shift

Since the pKa of the imine of the target system is lower than that of the substrate amine, a pH shift (reduction in pH as conversion increases) over the course of the reaction is to be expected. In order to cope

with the pH shift, buffers are generally employed at lab-scale. The strength of the buffer dictates the buffering capacity. The pH shift of using a buffer of a certain buffering capacity can be calculated by using the Henderson–Hasselbalch equation. The pH shift expected can be calculated and compared to that of a buffer with a higher buffering capacity (Figure 6-1). It can be seen that a pH shift of 1 unit would be expected when as little as 80 mM of the substrate is converted. It is therefore critical to maintain the pH of the reaction in order to utilise the full capacity of the biocatalyst.

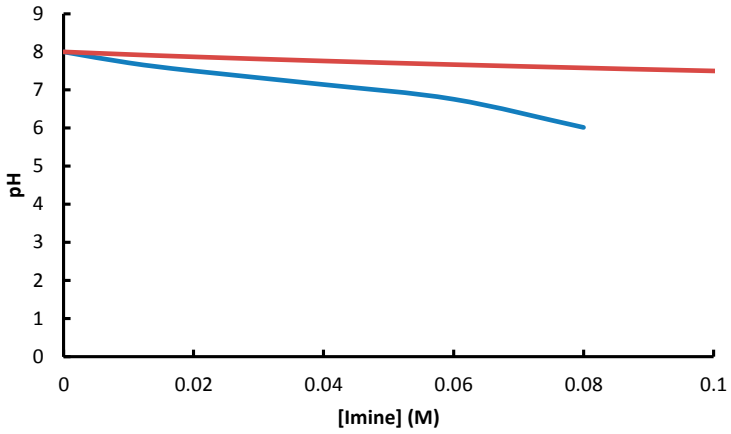


Figure 6-1: Shift in pH for 100 mM (blue) and 500 mM (red line) phosphate buffer as imine is produced

Overcoming pH shift

In a laboratory-scale preparation, buffers are often used to maintain pH. However, at scale, pH is commonly maintained by acid/base titration. However, to cope with the feed-back controls, the reaction is often started with a low concentration of buffer and pH maintained by titration. It is desired that high concentrations of acid and base are used for titration in order to avoid diluting the reaction mixture. However, this practice could lead to deactivation of the biocatalyst as some biocatalyst will be exposed to a very high concentration of base/acid for a short time (i.e. before mixing comes into effect)[144]. Therefore, the success of the method is often subjected to the mixing efficiencies and the biocatalyst stability to alkaline environment.

Although the pH shifts quite rapidly in a buffer with a low buffering capacity, the effect of the pH shift on the reaction depends on the pH profile of the biocatalyst. Therefore, the pH-activity profile was studied and a reaction was run at both 100 mM and 500 mM phosphate buffer and compared to understand the effect of the pH shift in the reaction.

In the previous sections, product-related limitations for the system have been identified to be product toxicity and pH shift (See also Chapter 5). In the following sections in this chapter, experiments were conducted to identify the extent of influence on these parameters through experimentation. Results and conclusions have been drawn and explained.

6.2 Materials and methods

All chemicals and reagents were bought from Sigma Aldrich unless specified and used as purchased.

6.2.1 Biocatalyst preparation

Plasmid

Plasmids (pET 16b vector) containing 3 different mutants of MAO genes derived from *Aspergillus niger* (wild-type)[52] were kindly donated by University of Manchester (Professor N. J. Turner).

LB broth and plates

LB broth was made with 10 g/L tryptone (Nordic biolabs AB, Täby, Sweden), 5 g/L yeast extract (Nordic biolabs AB, Täby, Sweden) and 10 g/L sodium hydroxide. To this, 2% (w/v) agar was added for making plates. The broth was autoclaved and ampicillin (filter-sterilized) was added to the broth (100 µg ampicillin/mL media) prior to use and the agar just prior to plating (100 µg ampicillin/mL media).

Preparation of competent cells

A starter culture was used to inoculate 5 mL of LB broth and the cells were incubated at 37˚C to grow the cells to an OD600 of approx. 0.6. 2 mL of cells were transferred to Eppendorf tubes and incubated in ice for 20min. The cells were then centrifuged at 6000 rpm for 10 min and the pellet was re-suspended in ice-cold water. The centrifugation step followed by re-suspension in water was repeated and centrifuged to obtain pellet. The pellet was re-suspended in 500 µL of 10% glycerol and centrifuged for 10 min. The supernatant was discarded, the pellet re-suspended in glycerol. The cells were stored as 100µL aliquots until further use.

Plasmid transformation

Plasmids (3 mutants) obtained from University of Manchester were transformed into competent *E. coli* BL21 by electroporation. To 50 µL of electro-competent cells, 0.5 µL of the respective plasmid was added and transferred to an electroporation cuvette (BIORAD, Copenhagen, Denmark) and exposed to a short pulse of high voltage. Following the electroporation, 950 µL of LB medium was added to the cuvette and incubated for 1h at 37 ˚C. 50 µL of the cell suspension was plated onto a LB plate containing ampicillin.

A colony was picked from the transformation plate, streaked onto a new plate and, incubated at 37 ˚C overnight. 5 mL of LB broth containing ampicillin was inoculated with a colony and allowed to grow to an OD600 of 0.6. This culture was then stored in 10% glycerol to obtain glycerol stocks. The stocks were stored at -80 ˚C until further use.

Production of biocatalyst

LB plates and pre-culture

From the glycerol stock, cells were streaked onto an LB plate containing 100 µg/mL ampicillin. The plate was incubated at 37 ˚C overnight. 5 mL of LB broth containing 100 µg/mL of ampicillin was inoculated with a colony from the LB-Amp plates. The culture tube was incubated at 30 ˚C in a shaker at 150 rpm for about 4 hours.

Fermentation

All fermentations were carried out in un-baffled shake flasks. To 500 mL of LB broth containing ampicillin, 5 mL of pre-culture was added (1% inoculum). The flask was incubated at 30˚C, 150 rpm. Cells were harvested after 18 h of growth by centrifugation at 4000 rpm, 20 min. The harvested cells were re-suspended in 100 mM phosphate buffer containing mono and di-basic potassium salts at pH 7.6 (25 ˚C) and used for biocatalysis. Fermentation was carried out prior to each biocatalytic reaction.

6.2.2 pH shift

pH-activity profile

Phosphate buffer with different ratios of mono- and di-basic potassium phosphate salts were prepared to obtain buffers at pH 6.5, 7, 7.7 and 8.2. Biocatalysis was carried out in baffled shake-flasks with a final working volume 20 mL of reaction mixture (2.9 g/L substrate and 5.6 gcdw/L biocatalyst suspended in 100 mM phosphate buffer) incubated at 37 ˚C and 150 rpm. The substrate, aza-bicyclo-octane HCl was procured from AK Scientific, (Union city, CA, USA). The sampling frequency was t= 0, 15, 30, 45 min, 1 h, 2h, 4h, 6h and 24 h. Samples were prepared and analysed using GC[145]. The pH profiles were made using a single batch of cells.

Effect of pH control

A reaction with 14.8 g/L of substrate amine was converted using 5.6 gcdw/L of cells. The reaction was carried out in both baffled shake-flasks (with phosphate buffer of 100 mM and 500 mM, pH 7.8) and a small

reactor (MiniBio, Applikon purchased from Holm & Halby A/S, Brøndby, Denmark) that was equipped with a 6-blade Rushton turbine, and, a pH and temperature control unit. The reactor has an internal diameter of 13 cm, with a maximum working volume of 250 mL and contains 2 baffles. The reactor was maintained at 37 ^{o}C and stirred at 800 rpm. pH was maintained at 7.8 (comparable to shake-flask experiments) by base titration (with 5M NaOH). The progress of the reaction was tracked using a GC.

6.2.3 Test for presence of trimer and product volatility

Biocatalysis was carried out with a substrate (amine) concentration of 2.96 g/L and biocatalyst concentration of 5.6 gcdw/L. The reaction was tracked over time by sampling and analysed using GC. The product solution obtained after conversion of all of the substrate was collected in a falcon tube and sonicated twice with 40% amplitude and 0.5 s cycles for 7 min. Following the sonication, cells were removed by centrifugation at 6000 rpm for 20 min. The supernatant was collected and used for testing product volatility.

20 mL of the supernatant was dispensed to baffled shake-flasks and incubated at 37 ^{o}C. To one of the shake flasks, *E. coli* cells were added (fermented in the same batch of cells as used for the biocatalysis). Samples were taken over a period of time and the product concentration monitored with GC. Similarly, 50 mL of the supernatant was poured in two stirred-tank reactor (MiniBio, Applikon, Holm & Halby A/S, Brøndby, Denmark) and to one of them *E. coli* cells was added. The reactor was maintained at 37 $^{\circ}$C. The product concentration was monitored. Additionally another experiment was set up where the reactor was filled with 50 mL of the product solution and nitrogen (set at an over pressure of 0.5 bar) was bubbled through the reactor.

Rotary evaporation

A reaction with a biocatalyst concentration of 5.6 gcdw/L and 14.8 g/L of substrate (amine concentration) was carried out. Complete conversion was verified by measuring substrate concentration with the GC. Following the reaction, the reaction mixture was sonicated and the cells separated by centrifugation at 4000 rpm for 30 min. To the supernatant 2 mL of 10 M NaOH was added. Following this, the supernatant was dispensed in 5 falcon tubes (each containing 10 mL) and extracted with 20 mL MTBE three times and the extracts were pooled together and evaporated in a rotary evaporator. The temperature of the rotary evaporator was set to 30 ^{0}C and pressure of 100 mbar was used. The condensate was collected and was analysed to test for traces of product. The solid obtained from evaporation was yellow crystals and were used for ^{1}H NMR analysis.

6.3 Results and discussion

Reaction species related constrains that were identified were product related. The product causes a pH shift in the reaction mixture, is potentially volatile and can form a trimer. Therefore, these constraints were studied in this Chapter and the results are discussed further.

6.3.1 pH optima for MAO

Phosphate buffer at different pH was used to study the pH optima. Since the pKa value of the buffer is 7.2, it is reasonable to use the buffer for testing the effect of pH at pH values between 6.2 and 8.2. Since the product produced would cause an acidic shift, the activity measurements were made only for pH at and below the reaction pH (~pH 8.0). Duplicates of the activity were measured and plotted (Figure 6-2). Activity of the biocatalyst at a particular condition was measured by obtaining the initial rate of the reaction over the first 30 min of the reaction. The results indicate that MAO has steep reaction optima where 50% of the activity is lost with a pH shift of 1 unit. These results correlate with an experiment made with MAO-B from both the microsome and mitochondria[146].

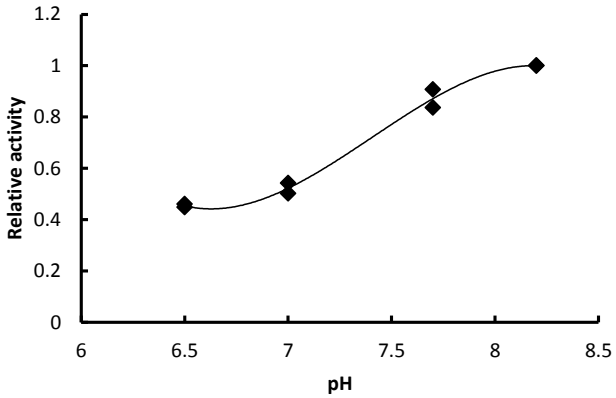


Figure 6-2: Effect of pH on biocatalyst activity. Substrate concentration 1.48 g/L, biocatalyst concentration of 5.65 gcdw/L. Duplicate experiments were performed and plotted.

6.3.2 Effect of pH shift on the reaction profile

From calculations of buffering capacity, it was inferred 500 mM buffer maintains the pH of the reaction mixture fairly well when converting substrate of a concentration of ~15 g/L. Therefore, in order to validate the effect of pH on the reaction profile, a reaction with 100 mM and 500 mM phosphate buffer was run and

the reaction profile was compared (Figure 6-3). The results indicate that the pH change which is quite significant in the 100 mM buffer causes a notable change in the final product concentration achieved. From Figures 6.3 and 6.4 we know that with 50 mM of imine produced, the pH is shifted by 1 unit and the activity of the biocatalyst is significantly reduced (approximately halved). These explain the deviation in the reaction profile between the two buffers.

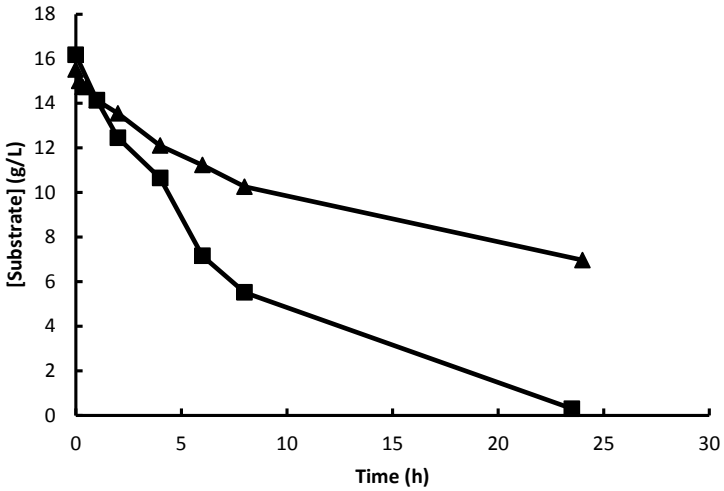


Figure 6-3: Comparison of reaction profile carried out in baffled-shake flask with 100 mM (▲) and 500 mM (■) phosphate buffer. Concentration of the biocatalyst was 5.65 gcdw/L

The shift in pH indicates that the pH control for the process has to be reliable. At large scale operations, this can particularly be a challenge because there might be zones of the reactor where the mixing is not homogeneous. Also, at the zone where the inlet for the base is placed, the cells will be exposed to high concentrations of the base for a short period of time which might cause deactivation. The inhomogeneity of pH and the effect of adding strong neutralising agents on the cells have been studied for fermentations[147,148], but this effect can be extended to biocatalysis as well. One should keep these considerations in mind when the process is developed.

A reaction was also run in a reactor where pH was controlled by titration of 10 M NaOH. The reactor experiments were conducted to compare the reaction profile obtained with a pH stat to that of a buffered shake-flask system (Figure 6-3). If catalyst deactivation by addition of base was present, it would be noticeable in the reaction conducted in the reactor. Additionally, changes in mass transfer between the stirred-tank reactor and the shake-flasks could be noticed in the reaction profile as well.

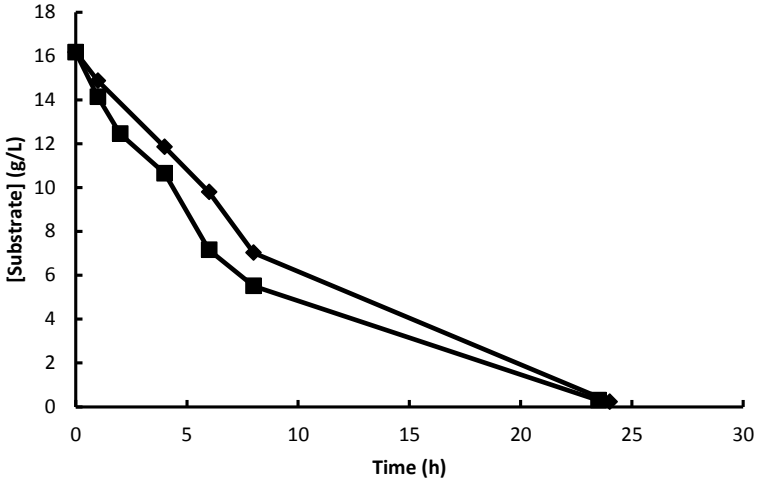


Figure 6-4: Biocatalysis performed with 5.6 gcdw/L in a buffered shake-flask system (■) and in a pH controlled stirred tank reactor (♦)

From Figure 6-4, it can be seen that the reaction profiles between the buffered shake-flask system and the reactor are similar indicating that the biocatalyst is not significantly deactivated during the reaction span. Also, mass transfer in the stirred tank reactor is similar to that of the shake-flask reactor.

These set of experiments indicate that the first product-related limitation to the system is pH shift and that the reactions should be carried out under a well-buffered system or in the presence of pH stat.

In order to test for the next identified product-related limitation, tests were carried out to test for the volatility of the product and will be discussed in the following section.

6.3.3 Product volatility and trimerization

A reaction carried out with 1.48 g/L of the substrate and a biocatalyst concentration of 5.65 gcdw/L was plotted as relative response of the substrate and product (Figure 6-5). Relative responses were plotted because the product could not be purchased and consequently, a standard curve for it could not be made. Also, in order to be able to compare the substrate and product in the same scale, both the substrate and product was represented it terms of their relative response obtained by the detection method. Relative response of the substrate is the ratio of the substrate to the area of the internal standard (1% PEA) and

similarly, the relative response of the product is the ratio of the product peak to that of the internal standard. Figure 6-5 indicates that there is a miss-match between the amount of substrate consumed and the product formed. The reasons for this could either be that the product is lost due to volatility, consumed by the cells or forms a trimer which is not traceable by the GC.

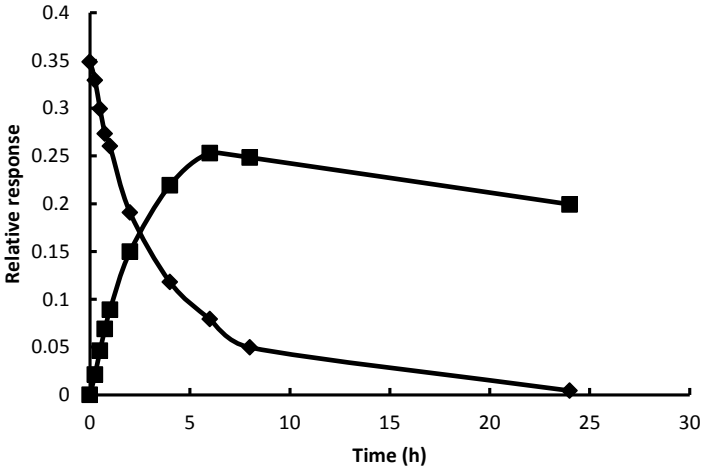


Figure 6-5 : Relative response of the substrate (♦) and product (■) plotted as a function of time. Relative response of the substrate is the ratio of the area of the substrate peak to that of the internal standard and similarly that of product is the ratio of the area of the product peak to that of the internal standard.

In order to check for product loss via volatility or by consumption to the cell, product solution was incubated in a 250 mL shake-flask flask (with 20 mL working volume) in the presence and absence of cells. The product concentration was monitored (Figure 6-6). Relative response was plotted and calculated as previously explained. A control of product solution was kept in a sealed falcon tube at room temperature and the product concentration was tracked at different time intervals.

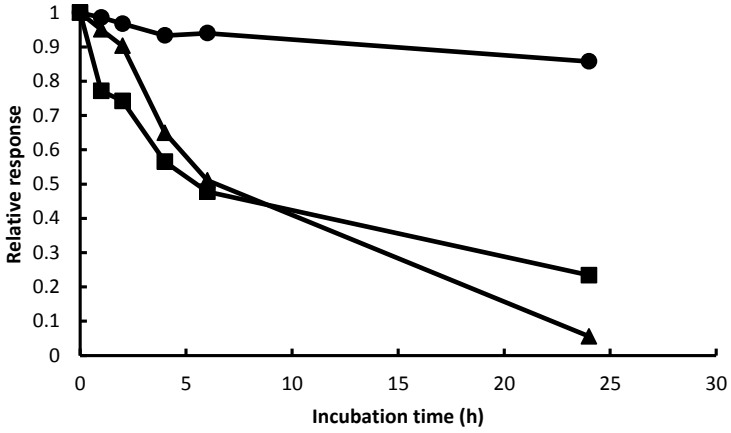


Figure 6-6: Test for volatility vs. consumption by the cells. The product solution was incubated in shake-flask with cells at 37 ˚C (■), without cells at 37 ˚C (▲) and incubated in falcon tubes at room temperature (●).

From Figure 6-6, it can be seen that the product profile with and without cells are very similar indicating that the consumption of the product by the *E. coli* cells is very unlikely. The product concentration reduces in a falcon tube maintained at room temperature but the reduction observed is not significant. This indicates that the product is not likely to be degraded over time.

In order to further test for volatility, the product solution was incubated in reactors and sparged with nitrogen. If the product was volatile, sparging with nitrogen will strip the volatile compounds from the reaction mixture. Since the flow rate of nitrogen was not measured, the rate of evaporation cannot be calculated. However, a comparison to the shake-flask experiments can still be made.

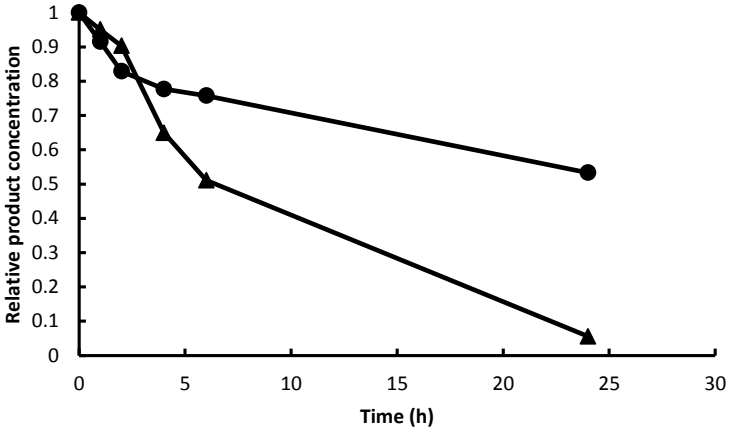


Figure 6-7: Test for evaporation of product in a reactor. The product solution did not contain cells. Nitrogen was sparged through with an over pressure of 0.5 bar (●) and a control experiment (▲) where the product was incubated in shake-flask is represented.

From Figure 6-7 it can be seen that the sparging nitrogen seems to have less impact on the product concentration than the shake-flask experiment (product concentration was found to reduce a lot more in the shake flask compared to the reactor). If the product was volatile, the volatility would be observed easier in a stirred reactor system with gas bubbling through it. This test therefore indicates that the product is not volatile. Additionally, in a different experiment where isolation of product was attempted using a rotary evaporator using reduced pressure, no product was observed in the condensate obtained after evaporation of excess solvent (MTBE) further indicating that the product is not lost in evaporation. It can also be seen from the predicted vapour pressure for the product (0.8 mmHg at 25 ^{o}C) that volatility can be neglected. From the above tests, volatility and product degradation by the cells can be neglected.

6.3.4 Trimer formation

Köhler and co-workers had shown that the product exists as a trimer and this is not traceable by the GC[57]. To confirm this, a ^{1}H NMR was made for the substrate, product and the reaction mixture after 4 h of reaction and 24 h of reaction (See Appendix VI). By doing this, the presence of the trimer and the extent of trimerization can be studied. The NMR confirmed the presence of a trimer. Integration of the peaks for quantitative response seemed unrealistic because several unidentified and overlapping peaks apart from the peaks corresponding to the substrate and product were seen. Further experimentation is required for confirming if these peaks are from the impurities from solvent and the salt used for product extraction or if they are degradation peaks from the product.

From the previous experiments, product trimerization has been confirmed. Polymerization of a similar imine has been reported previously[149]. It has been reported in this paper that the monomer and trimer exists in equilibrium and the equilibrium formation is almost instantaneous. In order to check for this, the reaction was run with different biocatalyst concentration and the relative response of the substrate and the product were plotted.

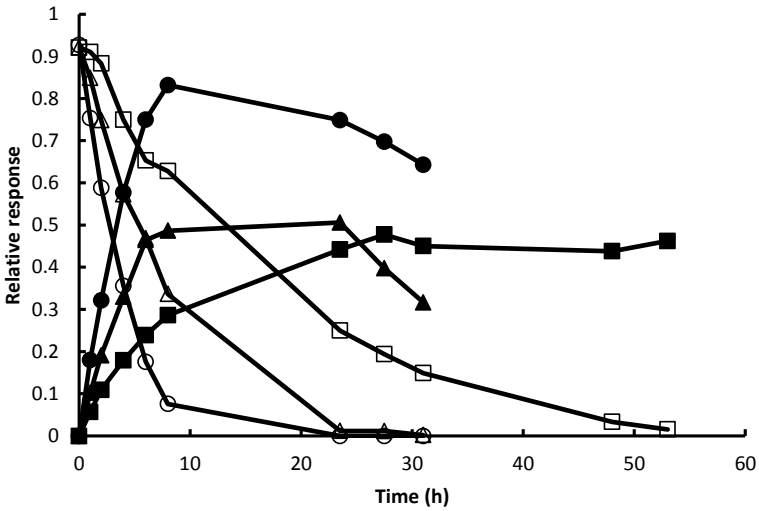


Figure 6-8: Relative response of the substrate (open symbols) and product (closed ymbols) plotted for biocatalyst concentrations of 2.5 gcdw/L (■), 5 gcdw/L (▲), and 10 gcdw/L (●). Substrate concentration for the reaction was 6.7 g/L. Relative response of the substrate is the ratio of the area of the substrate to the area of the internal standard. Relative response of the product is the ratio of the area of the product to the area of the internal standard

From Figure 6-8, it can be seen that the trimer formation does not seem to be instantaneous. If the reaction is fast enough (as in the case with the reaction with a biocatalyst concentration of 10 gcdw/L), all of the substrate converted is traceable as a monomer and then the trimerization begins. Additionally, trimer formation seems to be related to the presence of the substrate. The presence of the substrate seems to slow down the trimer formation (can be seen in reaction with 5 gcdw/L and 10 gcdw/L). This could perhaps be because the concentration of the monomer is driving the formation of the trimer.

6.4 Conclusions

In this chapter, product-related limitations have been identified. pH shift has been confirmed and this affects the final conversion achieved in a batch. pH maintenance by pH stat seems to overcome this

limitation. The product was identified to be non-volatile and the formation of trimer has been validated through NMR analysis. The rate of formation of trimer seems to depend on the cell concentration.

7. Biocatalyst-related limitations – Characteristics of the biocatalyst

Summary

Redox biocatalysis is currently gaining focus because it offers exquisite selectivity using mild oxidants. This includes oxygen, which is also environmentally benign. However, it is often challenging to implement oxidative reactions at scale due to the low activity and stability of the biocatalyst under industrial conditions. By identifying the bottlenecks for a specific oxidation reaction, it becomes possible to prioritise development efforts in the critical areas, i.e, on biocatalyst development (e.g. improvement of expression levels), process development (e.g. improved oxygen supply, product removal strategies) or biocatalyst stabilization (e.g. through immobilization or directed evolution). This chapter describes the first efforts to characterize the target reaction system for potential process limitations.

The biocatalyst-related limitations that were identified in Chapter 5 were validated and quantified through experiments in this chapter. Suggestions for implementing a product removal strategy for overcoming inactivation of biocatalyst by the product have been discussed.

A modification of this chapter has been published (Paper I).

7.1 Introduction

To develop a process, it is crucial that it be characterised and its limitations and targets identified. This chapter indicates the first efforts towards characterisation of the target reaction system (represented in Chapter 2). A systematic approach has been used to characterize the biocatalyst-related limitations. First, different mutants expressing the enzyme were screened for and then the best mutant was adopted for identifying the mass transfer of the substrate and product across the cell membrane, inhibition (reversible) of the biocatalyst by the substrate and (co)product, and inactivation (irreversible) of the biocatalyst by the substrate and (co)product Also, the inactivation of the biocatalyst due to reaction conditions (in the absence of substrate or product) also affects the reaction and has to be assessed. These limitations are quantified in this chapter.

7.1.1 Mass transfer across the cell membrane

Cell membranes are designed in nature to allow the transport of nutrients into the cell and keep the toxic substances outside. Therefore, the cell-membrane is often referred to as being semi-permeable. However, this permeability is often a disadvantage for employing the cells for biocatalysis[150]. For a whole-cell based biocatalytic process the substrate must be transported within the cell and made available to the enzyme. Following this, the product formed from the enzymatic reaction needs to be transported outside the cell. If the substrate is unable to enter the cell, the reaction will not occur. Furthermore, the rate of the reaction catalysed by the enzyme and the rate of transport across the membrane play a central role to how fast the reaction occurs. If the rate of transport of the substrate is faster than the biocatalytic reaction, one could consider that there is no mass transfer limitation with respect to the transport of the substrate across the cell membrane. This is the ideal case. However, at times, the rate of substrate transport is impeded by the presence of the cell membrane. Therefore, substrate availability has to be evaluated.

Similarly, for the ease of recovery of the product, the product has to be transported outside the cell. The transport of product across the cell membrane becomes particularly important when the product is toxic to the biocatalyst or the cell.

7.1.2 Stability of the biocatalyst

Biocatalysis as mentioned earlier is being implemented in many industrial processes. However, one central challenge that prevails is to deal with the biocatalyst stability. Biocatalyst stability can be affected by process conditions (such as temperature, buffer composition and salt concentrations) and chemicals reacting with the biocatalyst (eg. inactivation by the substrate or the product). The loss in stability by these can be addressed through different solutions. While the stability to reaction conditions can be varied depending on the process conditions chosen (i.e by operating at a different temperature, pH etc.). Stability loss due to the chemical species of a said reaction can be improved by process technology (ISPR, feeding etc.).

Inhibition and Inactivation of the biocatalyst

One of the desirable properties of an industrial biocatalyst is that it is stable at high product concentrations[151]. The requirement of stability at high product concentrations arises from the need for achieving a certain concentration of the product in order to facilitate easy and cost-effective downstream processing. However, since most biocatalysts in nature operate at low concentrations of substrate and product, they are seldom tolerant to high product concentrations. Consequently, this leads to substrate and/or product inhibition and toxicity, which is common to many biocatalytic systems.

Substrate toxicity, if present, can easily be overcome by feeding the substrate. The by-product of the target reaction is hydrogen peroxide which can potentially cause inactivation of the enzyme[152]. It is therefore necessary to remove the peroxide as it is formed to maintain the enzyme stability and it is hypothesised that this can be achieved by the use of another enzyme, catalase (EC 1.11.1.6). Finally, product toxicity becomes a critical factor to overcome and strategies to overcome that is discussed in the following section.

7.1.3 Overcoming product inhibition and toxicity

As mentioned previously, in order to achieve high productivities required by the target biocatalyst, the reactions are operated in such a way to obtain high product concentrations. This is important to reduce the downstream processing cost of the process. Consequently, inhibition and toxicity of the biocatalyst by the product becomes inevitable.

Inhibition and inactivation of the biocatalyst by the product can be overcome by protein engineering or by adopting the so called *in situ* product removal strategies[119,122,153,154]. *In situ* product refers to a set of techniques adopted to remove the products in the site of the reaction. Several methods of product removal are available for use by a process engineer. While the techniques are known, it is often not straight forward to apply the strategies. A successful ISPR exploits the difference in the physiochemical properties of the product of interest from the rest of the reaction mixture. However, the substrates and products in most biocatalytic reactions have very similar physicochemical properties which complicate the implementation of ISPR. Additionally, the selectivity of the ISPR is dependent on its driving force (which is the concentration of the product) and the determining factor for separation of the molecules (for example, if resins are employed, the capacity of the resins, its selectivity towards the product over the substrate, the rate of adsorption of the product/desorption of the substrate with respect to the rate of the reaction are some of important factors). Consequently, concentration profiles of the substrate and the product (that needs to be removed) affects the ISPR efficiency.

For the target reaction system, the substrate and product molecules have different pKa values and therefore, this difference can be exploited in order to obtain a successful ISPR technique. It is possible to determine the charge of the substrate and the product molecules at a given pH based on their respective pKa values. Furthermore, a plot of the ratio of uncharged molecules can be plotted (Figure 7-1). As the substrate becomes uncharged, the ratio of the substrate to product becomes higher, when both the substrate and the product become uncharged, the ratio becomes 1 (pH> 13).

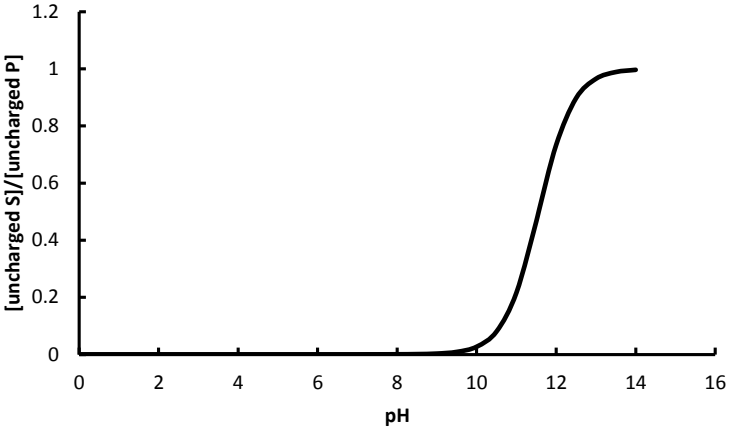


Figure 7-1: Ratio of uncharged substrate to uncharged product for the target reaction system as a function of pH.

Figure 7-1 indicates that at pH<10, the ratio of the substrate to product is almost zero indicating that most of the substrate is uncharged. However, it is only at pH 9 that all of the product molecules are uncharged (See appendix IV). Therefore, there is a flexibility of operating the ISPR at different pH (Figure 7-2).

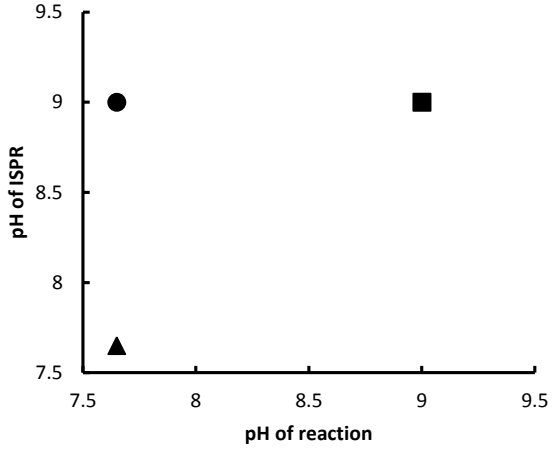


Figure 7-2: Flexibility in operating ISPR for the target reaction. The points represent the various options for operating the ISPR and the reaction. (▲) represents operating at pH optimum for the reaction, (■) represents operating at pH optimum for ISPR and (●) represents operating the ISPR at different pH.

As depicted in Figure 7-2, the pH of the ISPR can be set to the optimum pH for the biocatalytic reaction or the operated at the pH optimum for the ISPR. In both these cases, there is certain trade-off. The ideal

operation would be to catalyse the reaction at a pH that is optimum for the reaction and have an external loop for the ISPR where the pH can be adjusted to the pH optimum for separation. This would be operating the reaction at pH of 7.8 and the ISPR at pH 9 (represented by ● in Figure 7-2).

Since the charge is the separating factor, two of the most common ISPR techniques, i.e. solvent extraction and ISPR using resins can be employed[155,156]. When resins are used, the product is immobilized on the resins and thereby separated from the reaction mixture (from the site of the biocatalyst). Solvent separation involves a use of a two-phase reaction system where the solvent forms a separate organic phase and the biocatalyst is present in the aqueous phase. The reaction therefore takes place in the aqueous phase and the product molecules that are uncharged are extracted onto the solvent phase. However, solvent extraction may cause additional concerns with respect to the biocatalyst stability. Therefore, in this study use of resins for ISPR was tested.

The advantage of using a resin for absorption or adsorption (adsorption is a surface phenomenon, where the product gets attached to the resins through non-covalent bonding, while absorption occurs over the entire volume of the resin) is that the kinetics is rather fast. Moreover, the capacity offered by absorptive resins is higher compared to adsorptive resins. It was therefore chosen to operate a resin-based ISPR for this process. The resins can also be re-used after the extraction of the product. Several types of resins are available in the market for separation purposes.

In the following sections, the constraints of the system were outlined above were tested and quantified. The constraints were identified systematically where both reversible binding (inhibition) and irreversible binding (toxicity) of the substrates, product and co-product were studied. The results from this chapter form the basis for understanding that is required for analysing the process limitations for the target system for scale-up.

7.2 Materials and methods

All chemicals unless specified were purchased at Sigma Aldrich (Steinhiem, Germany) and used as purchased. The solvents were GC grade while the salts were analytical grade. Absorbent resins were kindly donated by Professor Daugulis (Queens University, Canada).

7.2.1 Plasmid

Plasmids (pET 16b vector) containing 3 different mutants of MAO genes derived from *Aspergillus niger* (wild-type)[52] were kindly donated by University of Manchester (Professor N. J. Turner).

7.2.2 LB broth and plates

LB broth was made with 10 g/L tryptone (Nordic biolabs AB, Täby, Sweden), 5 g/L yeast extract (Nordic biolabs AB, Täby, Sweden) and 10 g/L sodium hydroxide. To this, 2% (w/v) agar was added for making plates. The broth was autoclaved and ampicillin (filter-sterilized) was added to the broth (100 µg ampicillin/mL media) prior to use and the agar just prior to plating (100 µg ampicillin/mL media).

7.2.3 Preparation of competent cells

A starter culture was used to inoculate 5 mL of LB broth and the cells were incubated at 37˚C to grow the cells to an OD600 of approx. 0.6. 2 mL of cells were transferred to Eppendorf tubes and incubated in ice for 20min. The cells were then centrifuged at 6000 rpm for 10min and the pellet was re-suspended in ice-cold water. The centrifugation step followed by re-suspension in water was repeated and centrifuged to obtain pellet. The pellet was re-suspended in 500 µL of 10% glycerol and centrifuged for 10 min. The supernatant was discarded, the pellet re-suspended in glycerol. The cells were stored as 100 µL aliquots until further use.

7.2.4 Plasmid transformation

Plasmids (3 mutants) obtained from University of Manchester were transformed into competent E. coli BL21 by electroporation. To 50 µL of electro-competent cells, 0.5 µL of the respective plasmid was added and transferred to an electroporation cuvette (BIORAD, Copenhagen, Denmark) and exposed to a short pulse of high voltage. Following the electroporation, 950 µL of LB medium was added to the cuvette and incubated for 1 h at 37 ˚C. 50 µL of the cell suspension was plated onto a LB plate containing ampicillin.

A colony was picked from the transformation plate, streaked onto a new plate and, incubated at 37 ˚C overnight. 5 mL of LB broth containing ampicillin was inoculated with a colony and allowed to grow to an OD600 of 0.6. This culture was then stored in 10% glycerol to obtain glycerol stocks. The stocks were stored at -80 ˚C until further use.

7.2.5 Fermentation

LB plates and pre-culture

From the glycerol stock, cells were streaked onto an LB plate containing 100 µg/mL ampicillin. The plate was incubated at 37 ˚C overnight. 5 mL of LB broth containing 100 µg/mL of ampicillin was inoculated with

a colony from the LB-Amp plates. The culture tube was incubated at 30 ˚C in a shaker at 150 rpm for about 4 hours.

Fermentation

All fermentations were carried out in un-baffled shake flasks. To 500mL of LB broth containing ampicillin, 5 mL of pre-culture was added (1% inoculum). The flask was incubated at 30˚C, 150 rpm. Cells were harvested after 18 h of growth by centrifugation at 4000 rpm, 20 min. The harvested cells were re-suspended in 100 mM phosphate buffer containing mono and di-basic potassium salts at pH 7.6 (25 ˚C) and used for biocatalysis. Fermentation was carried out prior to each biocatalytic reaction.

7.2.6 Biocatalysis

Biocatalysis was carried out in baffled shake flasks with a final working volume 20mL of reaction mixture (substrate and biocatalyst suspended in 100 mM phosphate buffer, pH 7.6) incubated at 37 ˚C and 150 rpm. The substrate, aza-bicyclo-octane HCl was procured from AK Scientific, (Union city, CA, USA). The sampling frequency and duration varied depending on the experiment. Samples were prepared and analysed as described in the following section. Similar experiments were performed with the same batch of cells (to avoid variation between batches). In most reactions, unless specified, the biocatalyst concentration used was 5.6 gcdw/L.

Screening of MAO mutants

3 different mutants of MAO expressed in E. coli BL21 were used for biocatalysis with 2.96 g/L substrate (amine) and a biocatalyst (whole-cell) concentration 11.3 gcdw/L. Conversion after 6 h was measured using a gas chromatography according to sample preparation and chromatography protocol and compared. Conversion is obtained from the amount of substrate consumed in 6 h.

Mass transfer of substrate and product across the membrane

Two biocatalytic reactions with a substrate concentration of 6 g/L and biocatalyst concentrations of 13.5 gcdw/L and 20.5 gcdw/L were carried out at 37 °C. Samples were taken at different time points, and cells isolated from the reaction mixture by centrifugation at 4000 rpm for 2 min. Following this the supernatant was separated from the pellet. The samples from the supernatant were extracted as explained in the sample preparation section and analysed in the GC. Similarly, at the same time, another sample containing the reaction mixture including cells was extracted and analysed in the GC.

Substrate (amine) inhibition studies

Substrate (amine) inhibition was studied by carrying out the biocatalytic reaction at different substrate concentrations and measuring the initial rate and reaction progress. Initial rate was obtained by fitting a second order polynomial to the first 6 hours of the reaction and taking the derivative to this curve at t=0 min. In order to identify catalyst inactivation, 2 mL of cell suspension (~60 gcdw/L concentration) was added to the reaction mixture containing 5.92, 8.8 and 11.84 g/L of substrate after 24 h. Samples were taken at 3 h and 6 h after the addition of catalyst to monitor conversion.

Fed-batch reaction

A reaction with 2.96 g/L of substrate was initiated and 1.48 g/L of substrate was added step-wise at time t=2 h, 4 h, 6 h and 8 h. The reaction progress was monitored with GC and compared to a batch reaction with 60 mM substrate.

Product (imine) inhibition

In order to obtain product for product inhibition tests, a reaction with a substrate concentration of 2.96 g/L and a catalyst concentration of 5.65 gcdw/L was run to completion. The reaction mixture was sonicated to break open the cells. The cell lysate was stored at room temperature for a week prior to use. Different volumes of the reaction mixtures were diluted appropriately with phosphate buffer (100 mM, pH 7.6) to obtain various concentrations of product required for the inhibition studies.

Product (imine) toxicity

In order to test the inactivation of the biocatalyst by the imine, 0.27 g/L of the product was added to a cell suspension containing at a concentration of 5.65 gcdw/L. The mixture was then incubated at reaction conditions (37˚C, 150 rpm) for 24h. To this, amine (1.46 g/L) was added and the progress of the reaction monitored.

Also, biocatalyst at the concentration of 5.65 gcdw/L was exposed to product of concentrations 0.44 g/L and 0.87 g/L and was incubated at 37 $^{\circ}$C for 150 rpm. Reactions were started at t= 0, 2, 5 and 24 h by adding substrate such that the concentration was 2.96 g/L and the initial rate was measured. Relative activity was calculated and plotted.

Co-product (hydrogen peroxide) inhibition

3% hydrogen peroxide solution purchased from Sigma was used for these studies. The concentration of hydrogen peroxide in the solution was confirmed by spectrophotometric absorbance at 240 nm using a quartz cuvette. To the reaction mixture containing 2.96 g/L of the amine, hydrogen peroxide was added at concentrations of 2.5, 5, 20 mM and initial rate calculated as explained previously.

Co-product toxicity

For testing the inactivation of MAO by hydrogen peroxide during the course of the reaction, catalase from bovine liver was added to reaction mixture containing 14.8 g/L of substrate catalysed by both whole cells and crude extracts such that the final concentration of catalase was 2.5 mg/mL.

Preparation of crude extract

Following fermentation, the cells were harvested by centrifugation as mentioned previously. The cells were then re-suspended in potassium phosphate buffer (100 mM). The cell suspension was sonicated in an ice bath at an amplitude of 20% with 0.5 second cycles for 7 min. The extract was directly used for biocatalysis after sonication.

Biocatalyst stability at reaction conditions

Whole-cells from fermentation was re-suspended in 500 mM phosphate buffer and dispensed in baffled-conical flasks such that the final concentration of the biocatalyst would be 5 gcdw/L. The conical flasks were incubated at 37^{o}C and 150 rpm for 0, 24, 48 and 52 hours. Substrate was added at these time points such that the concentration of substrate in the shake-flasks was 2.96 g/L and the reaction followed by GC.

Test for oxygen limitation

In order to test for oxygen limitation, a reaction containing 14.8 g/L of substrate was catalysed with whole cells at concentrations of 1.25, 2.5, 5.65, 11.3 and 16.9 gcdw/L.

ISPR using absorbent resins

The resin capacity was evaluated by incubating the product in the presence of different concentrations of the absorbent resin (1, 3, 5 % (m/v)) at a pH 7.7 and at pH 9.5. GC was used to analyse the product concentration in the solution.

To test for the ISPR during the reaction, resins at a concentration of 10% (m/v) was tested in a batch set-up. The target biocatalytic reaction was carried out with a biocatalyst concentration of 5 gcdw/L and 100 mM substrate concentration and pH was maintained at 7.7 with 500 mM KPi.

7.2.7 Sample preparation

200 µl of reaction mixture was added to an eppendorf tube containing 10 µL of 10 M sodium hydroxide and 100 µL of 1% (v/v) 1-phenyl ethylamine (PEA) (Merck, Germany). To this, 1 mL of methyl tert butyl ether (MTBE) was added, vortexed for ~20 seconds and centrifuged (Eppendorf, Horsholm, Denmark) at 14100 rpm for 5 min. The supernatant was then transferred to another eppendorf tube and dried with sodium sulphate. This was then vortexed and centrifuged. 200 µL of the organic phase was then transferred to GC vials for analysis.

7.2.8 Gas Chromatography

The organic substrate and product were analysed in a J&W CAM column (Agilent Technologies, Horsholm, Denmark) by Clarus 600 GC-FID (Perkin Elmer, Skovlunde, Denmark) with a split injection, using a split ratio of 50:1. An isothermal method with column, injector and detector temperatures of 110 ˚C, 250 ˚C and 250 ˚C, respectively, was used. The carrier gas (nitrogen) flow-rate was set to 1.6 mL/min. Flow rates of 350 mL/min and 45 mL/min were used for the detector gases air and hydrogen, respectively.

7.3 Results and discussion

7.3.1 Mass transfer across the membrane

By isolating the supernatant and the cell fractions during the biocatalytic reaction and analysing each of these fractions for the substrate and the product, the partitioning of the substrate and the product can be identified.

Partitioning of the substrate

Partitioning of the substrate inside and outside the cell has been quantified for two different cell concentrations and plotted (Figure 7-3).

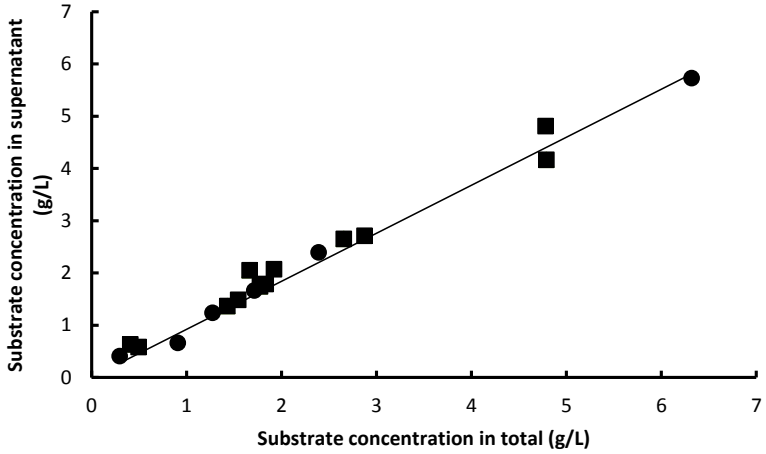


Figure 7-3: Represents the fraction of substrate in the supernatant for reactions catalysed by cell concentrations of 13.5 gcdw (■) and 20.25 gcdw/L (●). Duplicates for each cell fraction are plotted.

Figure 7-3 was obtained by measuring the distribution of the substrate during the course of the reaction. From the figure, it can be seen that the irrespective of the cell concentration, approximately 10% of the substrate is present in the cell fraction and 90% is present in the supernatant. Therefore, at the current situation, the rate of the reaction is lower than the rate of transport of substrate across the cell membrane. However, if the activity of the enzyme or the expression level of the protein is increased it is possible to observe a mass transfer limitation. Therefore, it is probably advantageous to operate the system without a cell membrane. However, the integrity of the cell membrane would contribute to the stability of the biocatalyst and need to be assessed.

Partitioning of the product

Similarly product distribution in the cell fraction and supernatant can be plotted. However, for this case study as depicted in Chapter 5, only the monomeric part of the product can be analysed by GC. Therefore the plot depicted here is a representation of only part of the product formed. Nevertheless, an idea about the distribution of the product can still be gained. From Figure 7-4, it can be seen that the product concentration in the supernatant is only about half the total product concentration. This indicates that about 50% of product is associated with the cell fraction and this is irrespective of the cell concentration. This result is similar to what has been observed in another *E. coli* system expressing a monooxygenase system[157].

A 50% distribution of the product in the pellet does not necessarily imply that it is present in the vicinity of the enzyme. Therefore product inhibition and toxicity values obtained from a whole-cell system are only apparent in the sense that the absolute values are not predictable.

Also, if product removal techniques are introduced, it might be desired that the ISPR is operated in conjunction with a biocatalyst which is either in the form of a lyophilized cells or as free enzymes. With either of these cases, the cost of the biocatalyst is a little higher than the cost for whole-cell operation. However, the benefits obtained (higher final product concentration) might be able to justify the additional cost. A cost-benefit analysis would be useful to assess the catalyst formulation required for this process.

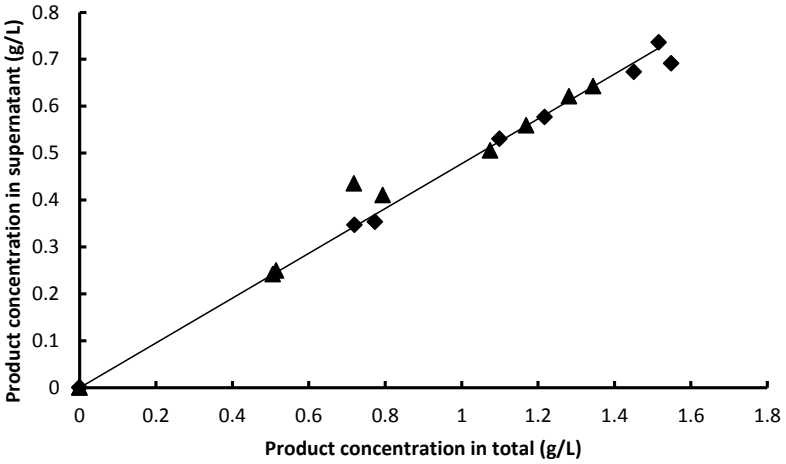


Figure 7-4: Representation of the fraction of total monomeric product molecules present in the supernatant for cell concentrations of 13.5 gcdw/L (▲) and 20.5 gcdw/L (♦).

7.3.1 Inhibition by the substrate (amine)

Reaction progress curves were obtained for the reaction and were used to obtain the initial rates at different conditions. While the extent of the reaction gives an idea about the reaction equilibrium and gives an insight about the presence/absence of toxicity to the biocatalyst (Figure 7-5), the initial rate gives an idea about substrate inhibition (Figure 7-6). Thermodynamic equilibrium is not expected to be limiting in this reaction since complete conversion for reactions at low substrate concentration is often observed (Figure 7-5). In these studies that initial rate measurements were made over the first 45 minutes of the reaction. Samples were taken at t=0 min, 10 min, 20 min, 30 min and 45 min.

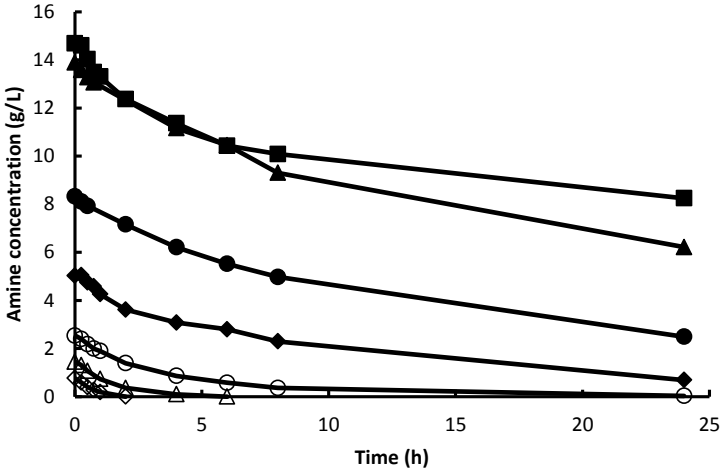


Figure 7-5: Reaction progress curves for MAO-N-D5 catalysed reaction at different substrate concentrations. (▪) – 15 g/L, (▲) – 14 g/L, (●)- 8 g/L, (♦)- 5 g/L, (○) – 2.5 g/L, (Δ) – 1.5 g/L, (◊) – 0.78 g/L.

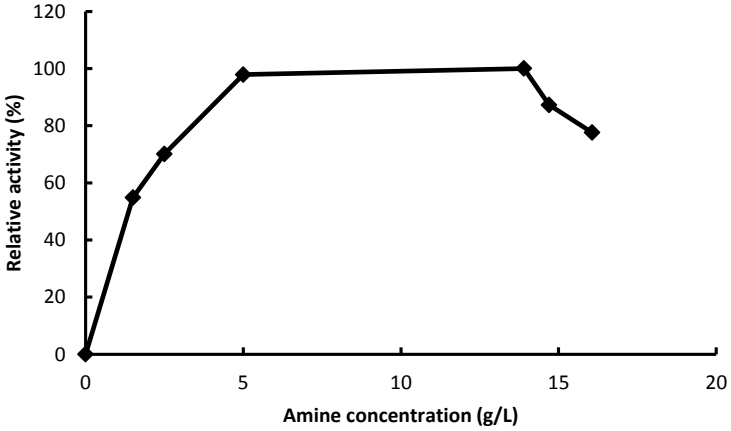


Figure 7-6: Initial rate for reactions at different substrate concentration.

From the initial reaction rates (Figure 7-6), it can be observed that substrate inhibition (caused by amine) becomes apparent at concentrations above 5 g/L and at a concentration above 14 g/L, it is seen to be more severe. This is in accordance with toxicity calculations that were made for the amine using the method described by Straathof (described in Chapter 5)[116]. Although the authors argue that the primary reason for

product toxicity is membrane disruption, this is not seen to affect the target reaction (as seen from the reaction profile using crude lysate in Figure 7-9). The authors also state that there are other causes for toxicity which involve specific effects such as inactivation of enzyme by toxic compounds. Therefore, it is interesting that this method could be used for predicting the toxicity effects in this system considering membrane disruption was not the source of the toxicity. Since the methodology, by its assumptions is limited to substances with an aqueous solubility of up to 0.2 M, this methodology was only tested for the substrate. The critical concentration is calculated on the basis of the aqueous solubility (52.45 g/L for the amine), and its logP (0.74). The solubility of the imine was calculated using the EPI software from EPA (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm).

From Figure 7-5, it can be seen that when the substrate concentration is higher than 5 g/L, complete conversion is not achieved in 24 h. A sample taken after 30 h of starting the reaction indicated no further conversion (results not shown). Addition of more catalyst allowed the reaction to further proceed indicating that the reaction had stopped due to a loss in catalytic activity (Figure 7-7). It is interesting that the conversion rate when fresh catalyst is added is slower and we suspect that the reason for the same is the presence of product inhibition and/or an effect of pH change during the reaction (Figure 7-7). This result is in accordance with the one obtained by Codexis, where the authors also see that conversion is marginally improved when fresh catalyst is added[69]. The possible reasons for the loss of catalyst activity are inactivation by (i) substrate (amine) (ii) hydrogen peroxide (iii) product (imine) and (iv) inactivation due to process conditions.

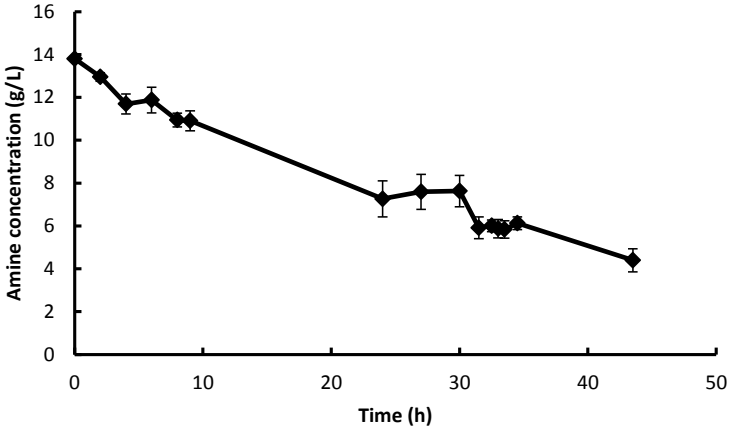


Figure 7-7: In a reaction run with an amine concentration of 14 g/L, the conversion has stopped after 24 h. This can be seen from the conversion points between 24 and 30 h. Addition of fresh catalyst at 31.5 h indicates that conversion is resumed albeit slowly.

7.3.2 Inhibition by product (imine)

Since the product (imine) is not commercially available, it needs to be synthesised prior to the inhibition tests. The product was synthesized as described in the materials and methods section. This method of imine synthesis was chosen because of its simplicity. Likewise, it circumvents the time consuming steps of purification and recovery (including extraction into solvent and drying with a rotary evaporator) that is involved in the chemical synthesis of the compound. In addition, when the product is synthesised using the chemical route, the imine obtained is in the form of a trimer which is not soluble in water. In order to solubilize the trimer, an alkali of an equivalent strength needs to be added to enable dissolution. It is therefore advantageous to produce the imine by biocatalysis as the product solution can be used directly after production. The cell suspension was sonicated to release the product from within the cells and the suspension was stored at room temperature for a week to ensure removal of residual MAO activity. The initial rate was used as an indication of product inhibition (Figure 7-8). The initial rate was obtained by following the substrate conversion since the product can co-exist (in equilibrium) as a monomer and a trimer, consequently complicating the analysis. Trimer formation has also been observed for another similar pyrroline molecule[149]. From the figure, it can be seen that the biocatalyst is inhibited by the imine with almost 50% of the activity being lost at a concentration of 0.7 g/L.

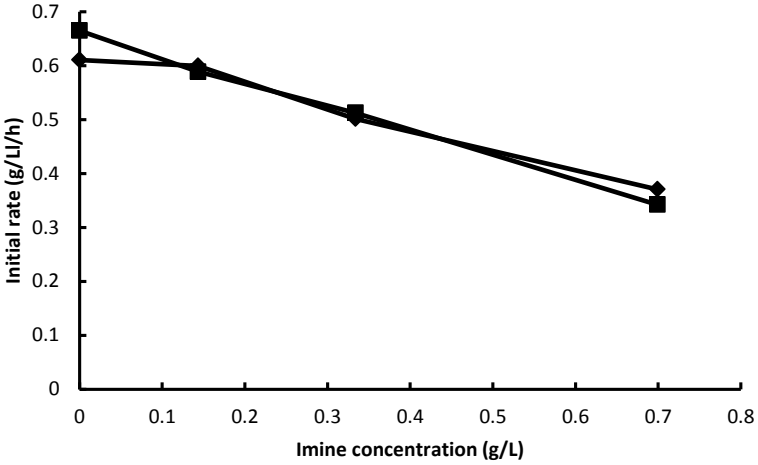


Figure 7-8: Initial rates for reactions at different product concentrations. The graph represents duplicates made with the same batch of biocatalyst.

7.3.3 Inhibition by co-product (hydrogen peroxide)

Inhibition studies using hydrogen peroxide indicated that there is a reduction in the initial rate when hydrogen peroxide is added to the system (Figure 7-9). It is also noteworthy that the reduction in reaction rate is very low (i.e from 0.92 g/L/h to 0.83 g/L/h). Also, the reaction progress (for reaction containing 2.9 g/L of substrate) indicated that the reactions proceeded to completion at the end of 24 h. This indicates that inhibition by hydrogen peroxide is present, at the conditions tested, but because the cells contain catalase, the MAO is stable enough to facilitate the reaction to completion. The presence of catalase was qualitatively assessed when hydrogen peroxide was added to the cells causing effervescence (caused due to production of oxygen when hydrogen peroxide is degraded). As to whether this is a function of catalyst turn over number (inherent to the number of active sites of catalase in the cells) remains to be tested.

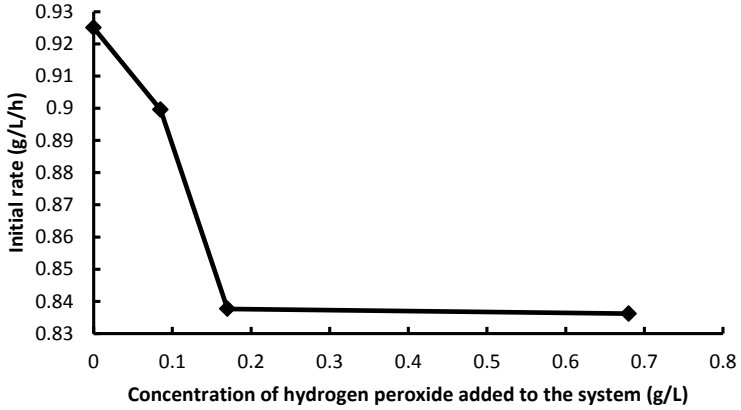


Figure 7-9: Inhibition by hydrogen peroxide.

7.3.4 Biocatalyst stability (Inactivation of the biocatalyst)

As can be seen from Figure 7-7, the catalyst is inactivated (irreversible loss in activity) after 24 h when converting substrates at concentrations over 5 g/L. The reason for the inactivation could be due to the substrate(s) and/or the product(s) and the inherent stability of the biocatalyst. Since oxygen is not supplied by sparging (but supplied by head-space aeration), shear forces that can be caused by gas-liquid interface is minimal. Hence, inactivation by oxygen is neglected. Inactivation by the other components (hydrogen peroxide, amine and the imine are discussed further).

Stability of the biocatalyst at reaction conditions

Biocatalyst stability could be influenced by reaction conditions (temperature, pH etc.) and the following section describes the results from stability studies carried out when no substrate was present over a period of 52 hours and the remaining activity measured by adding substrate to the reaction mixture. Since there was evaporation, the substrate concentrations at the start of the reaction was slightly higher in the flask incubated for 52h compared to the one where the substrate was added at t=0. This made the initial rate comparison faulty. Therefore, the substrate consumption profile was compared instead of just the initial rate (Figure 7-10). Comparison of the profiles indicates that there is no loss in stability at reaction conditions for 52h when no reaction is catalysed (Figure 7-10).

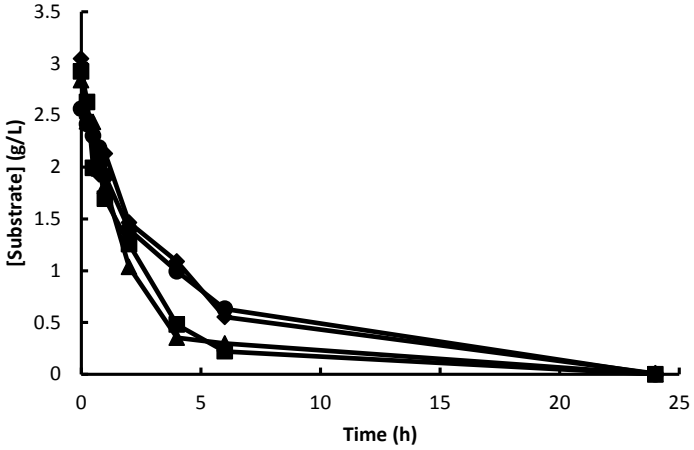


Figure 7-10: Stability of the biocatalyst at reaction conditions (37 °C, 150 rpm). Catalyst concentration of 5.65 gcdw/L (●) – No exposure, (▲) – 24h, (■) – 48h, (♦) – 52h exposure.

Inactivation by hydrogen peroxide

If the inactivation of the biocatalyst was caused by the hydrogen peroxide, removal of hydrogen peroxide should allow the reaction to proceed further. This can be achieved by adding an excess of catalase to the reaction system.

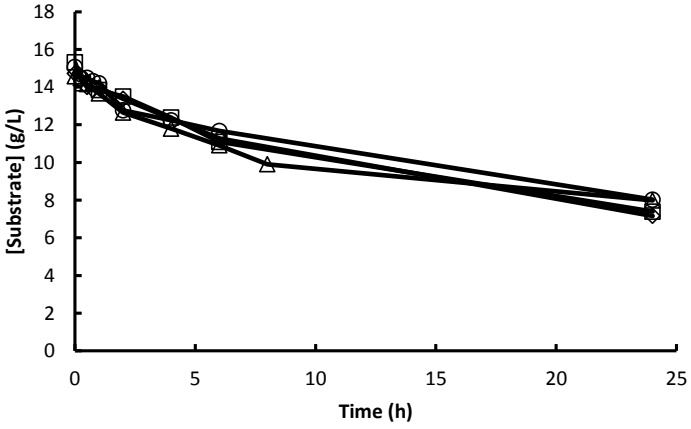


Figure 7-11 indicates the effect of adding catalase to the reaction mixture to degrade the hydrogen peroxide produced during the reaction. This was tested with reactions catalysed by both whole-cells and

crude enzymes to eliminate concerns regarding the transfer of catalase into the cell (to the proximity of hydrogen peroxide). The results indicate that the degree of reaction does not improve by the external addition of catalase indicating that (at this concentration) hydrogen peroxide is not causing inactivation of the cells. This could be attributed to the presence of catalase in the *E. coli* cells. This result implies that the inactivation is caused by either the substrate or the product and further investigation is required in this regard.

It is also noteworthy that the crude extracts and the whole cell reactions follow the same trend indicating that the mass transfer across the cell membrane is not limiting. The reaction is slower than the mass transfer rate across the membrane.

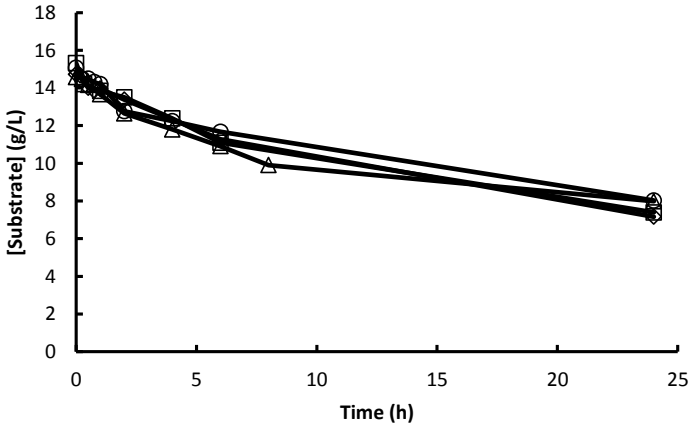


Figure 7-11: Effect of catalase on the reaction progress. Substrate concentration: 15 g/L, Biocatalyst concentration: 5 gcdw/L, catalase concentration – 2.5 mg/mL. (◊) – whole-cells (control), (Δ) – crude extract, (□) – whole cell with catalase, (○) – crude extract with catalase.

Inactivation by substrate (amine)

Inactivation by the exposure to the amine is quite tricky because it is not possible to incubate the catalyst in the presence of oxygen and at the same time prevent the reaction from proceeding. Diluting the reaction mixture and adding competing amine substrates was considered. However, such a method makes it difficult to separate the effects of product inhibition (and inactivation) from substrate inactivation. Inactivation by substrate could be tested by incubating the enzyme in the presence of the substrate in the absence of oxygen (by sparging nitrogen). However, sparging might cause shear effects due to gas-liquid interface and

interfere with the results. Thus, a different method of estimating the substrate toxicity was adopted. We argue that if substrate inactivation was present, fed-batch addition of the amine would help maintain the stability of the biocatalyst. It is for this reason, a fed-batch reaction was run and the product profile compared to that of a batch (Figure 7-12).

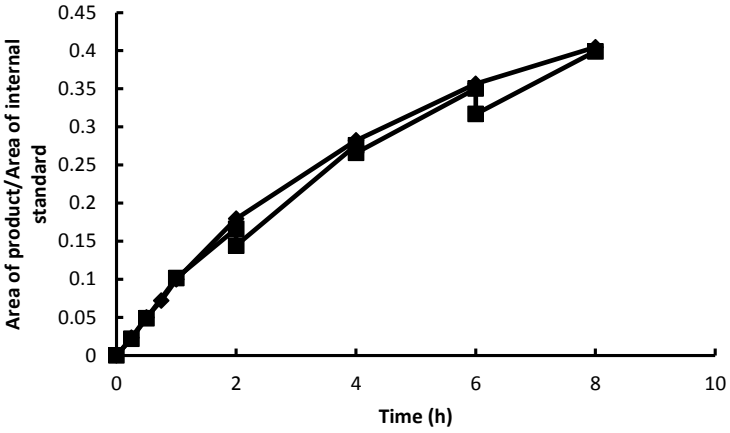


Figure 7-12: Stepwise feeding of the substrate to identify substrate toxicity. (■) represent fed-batch while (◆) represent batch reaction.

Figure 7-12 represents the ratio of area of the product to the area of the internal standard (sometimes known as relative response of the product) plotted against time. It can be seen that the profile of a fed-batch matches that of a batch reaction. This indicates that the inactivation of the biocatalyst is not due to the substrate. Furthermore, since the inactivation is not caused by hydrogen peroxide, substrate or the reaction conditions (pH, temperature), it can be attributed to the inactivation by the product.

Inactivation by product (imine)

Preliminary tests indicate that when the biocatalyst was incubated in the presence of 0.12 g/L and 0.17 g/L of the imine at reaction conditions (37 ˚C, 150 rpm) about 4% and 8% of the activity was lost respectively, confirming that the inactivation is indeed caused by the presence of the product. Additionally, the loss in activity of the biocatalyst increases with increase in duration of exposure further confirming inactivation of the biocatalyst. The inhibition by the cyclic imine product has also been reported in a study where a similar amine was used[69].

Figure 7-13 represents the relative activity of the biocatalyst when exposed to 4mM and 8mM of the product for different period of time (0 h, 3 h, 6 h, and 24 h). From the figure it can be seen that the relative

activity is reduced by approximately 33% and 44% when the cells are exposed to the product of 4mM and 8mM respectively for 24 h. Similarly, previous studies have shown that product toxicity is a function of both product concentration and duration of exposure[69]. In their study, the authors indicate a faster decay of the enzyme's activity when exposed to higher concentration of the product (The catalyst lost ~85% of its activity at a product concentration of 32 mM). For the target system studied in this thesis, the reaction is severely product toxicity limited and the final product concentration achieved will be a combination of speed of product production and the tolerance of the biocatalyst towards product toxicity.

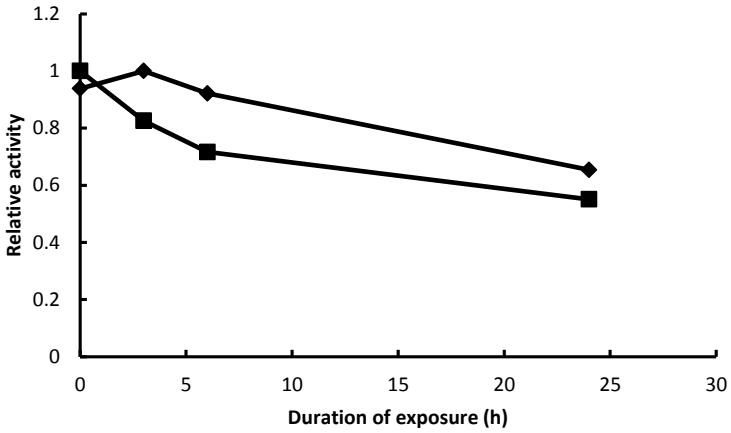


Figure 7-13: Figure represents the relative activity of the cells when exposed to the product for different durations of time. (♦) represents a reaction started with ~4mM product while (■) represents a reaction started with ~8mM of the product.

7.3.5 Overcoming product toxicity

In situ product removal technique using absorptive resins was suggested for removing the product. The separation technique employed was based on the fact that the charge of the substrate and the product is different at different pH (as explained previously). The capacity of the resins to absorb the product was first tested and the % of product absorbed was plotted. Product absorbed was traced by the product left in the solution. It can be argued that the absorption might be overestimated because this approach does not take trimerization into account. However, this method offers a quick screening technique for the success of implementation of the resins for product removal. Arguably, since only uncharged molecules will be absorbed by the resin, the trimer should also be absorbed but this was not validated. Also, since the monomer and trimer exists in equilibrium, the trimer will convert to monomer as absorption occurs.

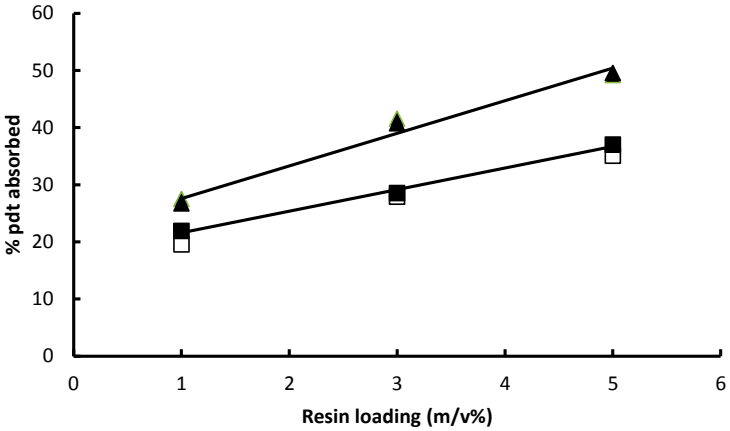


Figure 7-14: Test for resin capacity at pH 7.7 (■) and pH 9.5 (▲). Duplicate samples were taken and have been plotted.

From Figure 7-14, it can be seen that about 50% of the product gets absorbed onto the resin at pH 9.5. While the product absorption is lower at the pH that is optimum for the reaction (pH 7.7), it is not significantly lower. This indicates that the resins can be used for product recovery.

7.3.6 Test for oxygen limitation

The reaction rate for any biocatalytic reaction is affected by the concentrations of the substrate(s) and the biocatalyst. For the target oxidation reaction, this rate is affected by the concentrations of the amine, oxygen and the biocatalyst. If the amine concentration and the oxygen supply rate are kept constant, the reaction rate is solely affected by the biocatalyst concentration in the system.

The requirement of oxygen supply is set by the maximum rate achieved in the system during the course of the reaction. Initial rate of the reaction is considered here as the demand of oxygen supply is highest during the period over which initial rate is measured. By plotting the initial rate over various enzyme concentrations, one can determine the region where oxygen becomes limiting. When oxygen is not limiting, the initial rate linearly increases with the biocatalyst concentration, however, when oxygen becomes limiting, the rate is determined by the rate of oxygen supply and therefore levels off.

For the target system, since the rate of conversion of the substrate is rather low, oxygen was not expected to be limited. This was confirmed by performing experiments with increasing concentration of cells and measuring the initial reaction rate. The result shows a linear increase in the rate with the enzyme concentration (Figure 7-15), indicating that there is no oxygen limitation. The maximum oxygen transfer capacity for an un-baffled shake-flask has been shown to be around 10 mmol/h (corresponding to 1.48 g/L/h) at a shaking speed of 150 rpm, with a 26 mL filling volume[158]. For a baffled shake flask, this is expected to be higher. Therefore, it is not surprising that the reaction is not oxygen limited in these conditions. The result obtained is in contrast to the study reported by Codexis[69], where it was seen that the initial rate increased with the oxygen concentration.

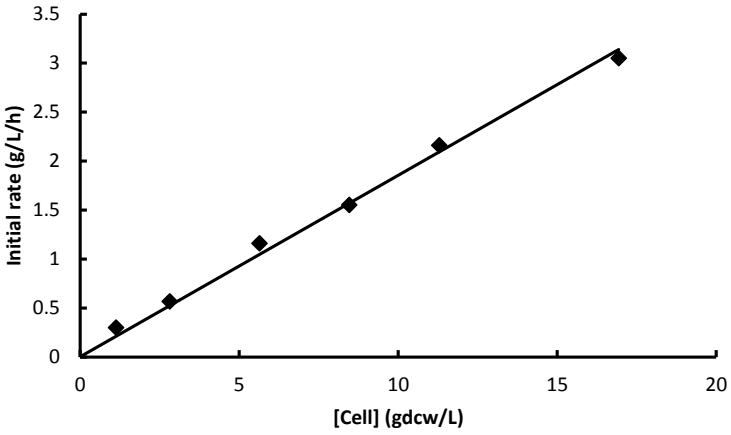


Figure 7-15: Reaction rate as a function of cell concentration at a substrate concentration of 6 g/L.

7.3.7 Constraints identified

From the above discussed results several constraints have been identified that prevent this system from being economically viable for large scale operation. The constraints identified are threefold (i) substrate inhibition, (ii) product inhibition (reversible loss of activity) and (iii) product toxicity (irreversible loss of activity). Likewise in a study by Zhang and co-workers[69], on the oxidation of a similar amine with a different MAO mutant, similar constraints were identified to the ones presented in this study. In both these cases the system was severely affected by product inhibition, although substrate inhibition (by the amine) seems to be a more serious issue in the Codexis example.

Targets for pharmaceutical products have been set based on the economy of the process. For example a typical pharmaceutical product it will be necessary to have a target product concentration of 50-100 g/L, a whole-cell biocatalyst yield of 5-10 g/gcdw and a reaction yield greater than 95%[127,159,160]. A typical fermentation yield for E. coli is 50 gcdw/L. Hence it can be expected that for this process production of biocatalyst will not be a limitation. In fact a target biocatalyst concentration has been set at 25 gcdw/L, based on our experience with difficulty in sampling when running biocatalysis at such high concentrations of cells. Likewise, oxygen supply is limited to 14.8 g/L/h (corresponding to 100 mmol/L/h) when air is used to supply oxygen to the reactor[97]. Although oxygen can be supplied at a higher rate (up to 59.2 g/L/h) when pure oxygen is used instead of air, this would incur additional costs and appears unnecessary. It should be noted that agitation and mixing in larger vessels might be worse and have to be accounted for successful scale up of oxygen supply.

Implications for oxygen requirements for the target system

Oxygen requirement in this type of biocatalytic system is the highest when the rate of the reaction is maximum since stoichiometric amounts of oxygen is required by the system. The maximum rate for a biocatalytic reaction is the initial rate and therefore a plot of the variation of initial rate would give an indication of the maximum oxygen requirement for the system. As mentioned earlier, the limit for oxygen supply is about 14.8 g/L/h.

Also, when the concentration of whole cells used in a reactor is higher than 20 gdcw/L difficulties in product recovery was observed further advocating the use of lyophilised cells. Therefore, these limits can be marked-off on Figure 7-15. By doing so, it is possible to obtain an operating region where the system is not oxygen limited (Figure 7-16). Such plots where a process constraint and process variable are expressed are commonly referred to as regime plots[161]. When the protein expression of the host system or the specific activity of the enzyme is improved, then the maximum rate achievable by the system increases. However, depending on the number of folds of increase, it is likely that the system would reach oxygen supply limits as expressed in Figure 7-16 (drawn based on the assumption that there is no mass transfer limitation of the substrate across the membrane). However, it is probably not critical that the process is operated at the oxygen limitation conditions for economic scale-up. Therefore, knowledge of the maximum rate that a process is required to operate at would make it easier to identify if efforts are required in terms of process development for improved oxygen supply.

For example, in this system, if a product concentration of 50 g/L is desired in a time period of 24 h, one could operate the system as it is with a maximum specific activity of ~0.2 g/g/h at a biocatalyst

concentration of ~10 gcdw/L. However, if the biocatalyst is affected by stability i.e it loses stability after a few hours of operation, then it is desired that the reaction rate is increased. In such cases, it is desired that improved oxygen supply strategies are adopted to increase the rate at which the product is formed.

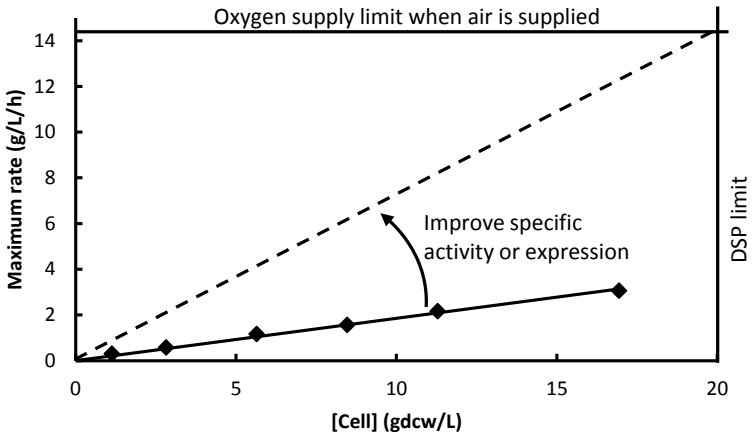


Figure 7-16: Regime plot for oxygen requirement to the system. Dotted line represents the improved maximum specific activity of the biocatalyst when the expression is improved.

From the biocatalyst-related limitations identified through experiments in this chapter, it is clear that use of whole-cell system for this specific case provide more challenges to the system. If the expression is increased, mass transfer of substrate would be limiting. Also, use of whole-cells makes product recovery difficult. Therefore, it is advisable to switch to the use of a cell-free extract or lyophilised cells for the process. It should be noted that the stability of a different biocatalyst formulation is likely to vary from using whole-cells and need to be evaluated.

Further limitations to the reaction system have been identified in context of the economics of the process in the following chapter.

7.4 Conclusions

Biocatalyst-related limitations were quantified in this chapter. This indicates that there is no mass transfer limitation across the membrane for the substrate while half the product is trapped within the cell. As a result, it becomes advantageous to use lyophilised cells or cell-free extracts for the process. Furthermore, at the rate at which the reaction proceeds, it is not limited by oxygen and is therefore not a concern at this

very moment. However, with an improvement in the protein expression, the situation is likely different and the focus will shift from the expression to potentially the combined improvement, oxygen supply as well as the means to keep the biocatalyst stable.

Part IV

8. Analysis of process limitations

Biocatalytic reactions are implemented to provide for the exquisite selectivity that the chemical counterparts cannot offer. However, implementation of the biocatalytic reactions at industrial scale is often a challenge in part due to the complex interaction between the reaction species and the biocatalyst. These interactions affect the activity and stability of the biocatalyst under industrially relevant conditions and consequently determine the economic feasibility of a process at scale. This chapter uses the results from the previous chapters where limitations to the target system were identified and uses this knowledge gained in conjunction with more experiments to make a more general assessment of the overall process to see which targets need to be met to make the process scalable.

8.1 Introduction

The success of a biocatalytic process is evaluated relative to the process targets such as biocatalyst yield or space-time yield, reaction yield and product concentration. The targets are set based on the market value of the substrate and product[162]. It is necessary that the process meets all these targets for a particular system.

The reaction yield of the process gives an idea about the efficiency with which the substrate is used. The reaction yield is stoichiometric when the substrate or product is not lost due to volatility. However, when the substrate or product is lost, or when the reaction suffers from thermodynamic limitations, the maximum reaction yield cannot be reached.

The product concentration which represents the product produced per volume of the reactor places demands on the downstream processing. Biocatalysis operates in water-based systems which are often dilute and involve the separation of a large quantity of water[163]. Consequently, downstream processing costs contribute significantly to the process costs. To make the process more economically viable, it is essential to have high product titre coming out of the biocatalysis reaction and it is therefore critical to understand and estimate the maximum product concentration achievable in a process at different catalyst concentrations.

Furthermore, since this process is based on non-metabolically active cells, the cost contribution from the biocatalyst (i.e. biocatalyst) becomes important. The cost contribution from the biocatalyst can be seen

from the biocatalyst yield. Biocatalyst yield is the amount of product produced per gram of biocatalyst and represents the biocatalyst cost contribution to the process.

Additionally, since it is an oxygen-requiring process, oxygen supply can be a process constraint and therefore the maximum rate achievable (initial rate) by the system at different catalyst concentration is an important metric.

The targets for the reaction system are set based on the market value for the product. Relative costs of the substrate to the product dictate the economics of the process. For the target reaction system considered in this thesis, the substrate can be purchased from AK Scientific at a cost of $5 per gram. Arguably a bulk purchase of the substrate could result in a cost that is an order or two lower. However, the product cannot be procured from the market. A quote for the synthesis of the product given by AK Scientific was $ 400 per gram. For the sake of process evaluation for economic feasibility, it is required to first set targets for a particular reaction system. It is noteworthy that the value of the product will be determined by the market and has to be evaluated carefully. In the following sections, the product is assumed to be a high value product of pharmaceutical relevance.

Examples for targets for the economic metrics and are summarized for a pharmaceutical product in Table 8-1.

Table 8-1: Examples of targets for pharmaceutical product produced using whole-cell biocatalysis

Parameter	Examples of target
Product concentration	50 g/L[159]
Space time yield (STY)	>2.5 g/L/h[159]
Biocatalyst yield	10-35 gP/g dry cell weight[116,160]
Reaction Yield	>90%

For the target reaction catalysed by monoamine oxidase, the product toxicity and expression levels are identified as the potential (from experiments in Chapter 7). Most of the experiments conducted up to

Chapter 7 were carried out in a fixed catalyst concentration of 5 gcdw/L. However, changes in the reaction or process conditions would lead to changes in the performance of the reaction system. For example, by varying the catalyst and the substrate concentrations the effect of changing regimes on the process targets can be evaluated. That is, by varying the substrate and catalyst loads, further analysis of the impact of catalyst load and product toxicity to make an assessment of the potential for improvements in the process has been performed.

In order to visualise the limitations of a process and the improvements attained, 2-D plots called regime plots have been used[161]. With regime plots, it is possible to identify the gain attained by relaxing the limitations present to the system. Combining the regime analysis with sensitivity analysis (where the effect of changes in variables on process constraints can be studied) would in turn allow for further process understanding and help identify operating conditions for the system[164].

Once the limitations and the effects of removing them on the target metrics are identified, two of the most significant economic parameters (for the target system) can be plotted against one another. The targets for the economic metrics can be plotted and the improvements on limitations can be visualised (Figure 8-1).

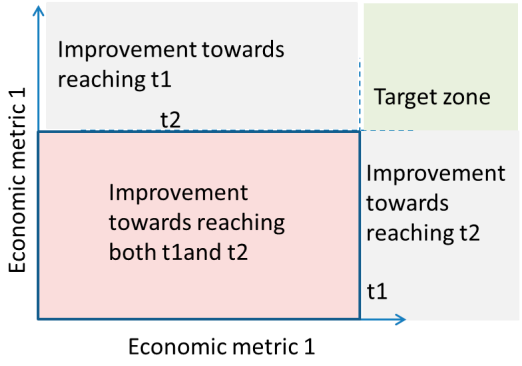


Figure 8-1: Plot of one economic target against another. t1 and t2 represent the targets for the economic metrics 1 and 2.

The following sections in this chapter will discuss experiments that were carried out to quantify the process limitations based on previous Chapters. Following this, a sensitivity analysis on removing the process constraints were made and finally plotted against the economic target to identify where the efforts for improvement need to be placed.

8.2 Materials and methods

All chemicals unless specified were purchased at Sigma Aldrich (Steinhiem, Germany) and used as purchased. The solvents used were GC grade while the salts were analytical grade.

8.2.1 Plasmid and fermentation

Plasmids (pET 16b vector) containing 3 different mutants of MAO genes derived from *Aspergillus niger* (wild-type) [Schilling & Berch, 1995] were kindly donated by University of Manchester (Professor N. J. Turner).

The plasmid was transformed into competent BL21 strains and fermented as explained in Chapter 4.

8.2.2 Biocatalysis

Biocatalysis was carried out in baffled shake flasks with a final working volume 20mL of reaction mixture (substrate and biocatalyst (in whole-cell formulation) suspended in 500 mM phosphate buffer, pH 7.6) incubated at 37 ˚C and 150 rpm.

The substrate, aza-bicyclo-octane HCl was procured from AK Scientific, (Union city, CA, USA). Samples were taken at t= 0, 1, 2, 4, 6, 8, 24, 27, 30, 48 and 52h. Samples were prepared and analysed as described in the following section. Similar experiments were performed with the same batch of cells (to avoid variation between batches).

The cells from fermentation was harvested by centrifugation at 4500 rpm for 20 min and re-suspended in phosphate buffer (used for the reaction) to achieve a concentration of ~50 gcdw/L. Dilutions of this suspension were used to achieve desired concentration for the reaction. Biocatalysis was carried out at different cell concentration (1.25, 2.5, 5, 10, 20 gcdw/L) to react different substrate concentrations (2.6, 7 and 14 g/L).

For biocatalysis using biocatalyst concentrations of 10, 15 and 20 g/L, the reaction was repeated and substrate (approx. 0.11 g) was added to the reaction mixture at 24h.

8.2.3 Sample preparation

200 µl of samples of reaction mixture were added to Eppendorf tubes containing 10 µL of 10 M sodium hydroxide and 100 µL of 1% (v/v) 1-phenyl ethyl amine (PEA) (Merck, Germany). To this, 1 mL of methyl *tert*-butyl ether (MTBE) was added. The tubes were vortexed for approx. 20 seconds and subsequently

centrifuged (Eppendorf, Horsholm, Denmark) at 14100 rpm for 5 min. The supernatant was then transferred to another Eppendorf tube and dried with sodium sulphate. This was then also vortexed and centrifuged in an equivalent manner. Finally, 200 µL of the organic phase was transferred to GC vials for analysis.

8.2.4 Gas chromatography

The organic substrate and product were analysed in a J&W CAM column (Agilent Technologies, Horsholm, Denmark) by Clarus 600 GC-FID (Perkin Elmer, Skovlunde, Denmark) with a split injection, using a split ratio of 50:1. An isothermal method with column, injector and detector temperatures of 110 ˚C, 250 ˚C and 250 ˚C, respectively, was used. The carrier gas (nitrogen) flow-rate was set to 1.6 mL/min. Flow rates of 350 mL/min and 45 mL/min were used for the detector gases air and hydrogen, respectively.

8.3 Results and discussion

The following sections discuss the results from more experimental work for identifying limitations, specifically product toxicity as a function of catalyst concentration. Furthermore, an analysis the impact of alleviating the limitations on the reaction system has been described.

8.3.1 Product-time profiles at different biocatalyst concentration

Product-time data were procured for different substrate and catalyst concentrations to understand process limitations such as loss in biocatalyst stability (which can be seen if no further conversion is achieved at substrate non-limiting regimes). For the target reaction system the maximum theoretical yield based on stoichiometric conversion is 0.7 g product per g substrate. The yield appears to be low, but this is a result of using a hydrochloric salt as a substrate making the molecular weight of the substrate 148 g/mol. Figure 8-2 represents the product-time profiles for reactions catalysed at different substrate and product concentrations. The product concentrations plotted here are calculated from substrate conversion, assuming stoichiometric yield (i.e. 1 mol of substrate forms 1 mol of product).

It can be seen that at low substrate concentration (Figure 8-2a), the maximum product concentration achievable is limited only by the duration of the reaction, that is, all of the substrate is converted to product as long as enough time is provided for the reaction. At increasing substrate concentrations, full conversion is not consistently achieved (Figure 8-2b, Figure 8-2c). For high enzyme concentrations (>10 gcdw/L), all the substrate is converted to product. Figure 8-2c indicates that the conversion for biocatalyst concentration of 1.25 and 2.5 gcdw/L has stopped after 30 h.

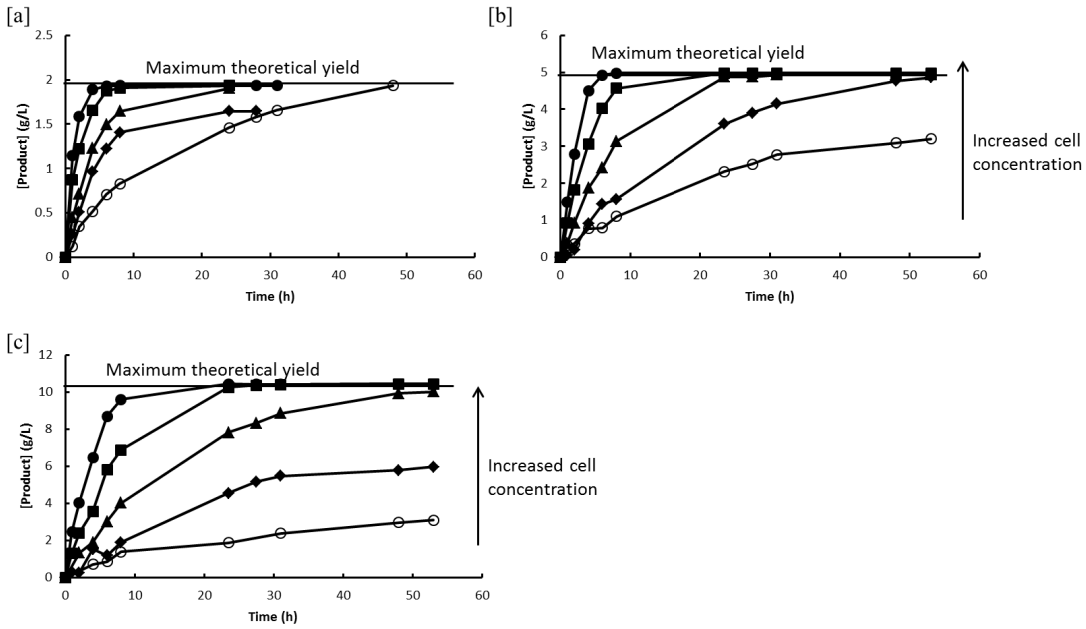


Figure 8-2: Product profiles at different enzyme (●) – 20 gcdw/L, (■) – 10 gcdw/L, (▲) – 5 gcdw/L, (♦) – 2.5gcdw/L and (○) – 1.25 gcdw/L and substrate concentrations (a) 2.6g/L, (b) 7 g/L and (c) 14 g/L.

Based on the final yields represented in Figure 8-2, the achievable reaction yields can be plotted as a function of cell concentration (Figure 8-3). In all reactions represented in Figure 8-2, complete conversion of substrate was observed at high biocatalyst concentration (biocatalyst concentration ≥10 gdcw/L) (Figure 8-3). In order to check for the activity remaining in the biocatalyst after complete conversion of the substrate, when the substrate is not limited (at cell concentration > 10 gcdw/L), substrate was added to the flask at the end of 24 h. The substrate added was in solid form and then the consumption was traced until no further conversion or all of the substrate was used was tracked (Figure 8-4). In the reaction with a biocatalyst concentration of 10 gcdw/L, it was observed that no further conversion was seen (after 48 h) indicating that the biocatalyst lost all activity. But with a cell concentration of 15 and 20 gcdw/L, most of the substrate was used. Therefore, the final product concentration achieved cannot be assumed to be the maximum possible product concentration achieved at these biocatalyst concentrations.

For obtaining the final product concentration achieved by the target reaction system at high cell concentrations, the substrate has to be fed in such a way that the substrate concentration is below the

toxicity limit. Furthermore, the biocatalyst is affected by a time-dependant product inactivation (as seen in Chapter 7). As a result, the method adopted for feeding the substrate becomes important for the product concentration achieved. Therefore, though a step feed has been adopted in this study, it is likely not the best strategy for using the entire potential of the biocatalyst. Continuous feeding would prove more advantageous in such scenario.

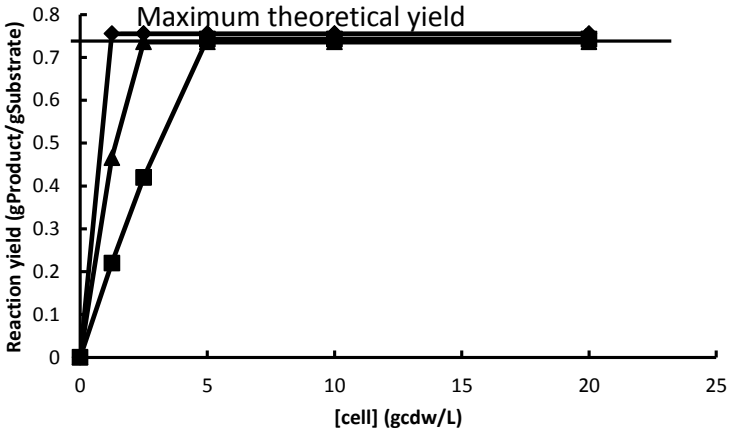


Figure 8-3: Reaction yield plotted as a function of cell concentration for reactions started with a substrate concentration of (♦) 2.6 g/L, (▲) 7 g/L and (■) 14 g/L.

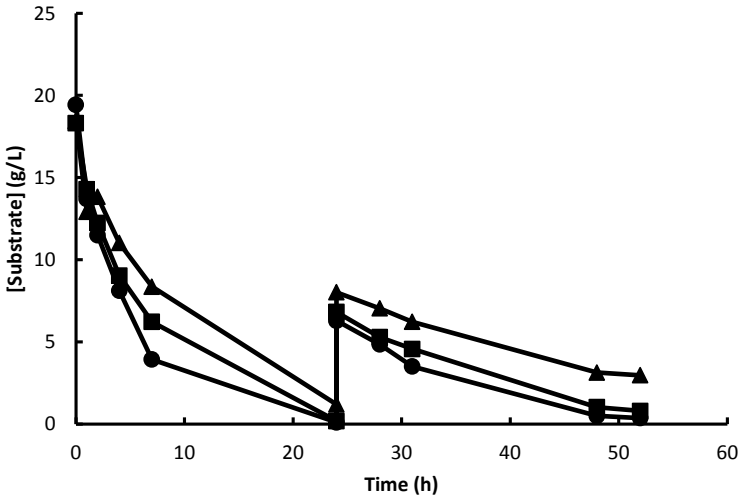


Figure 8-4: Reaction catalysed by biocatalyst at concentrations of (▲) – 10 gcdw/L, (■) – 15 gcdw/L and (●) – 20 gcdw/L. The reaction was fed with a substrate concentration of approx. 10 g/L at 24h.

8.3.2 Reaction rate and oxygen transfer limit

The results shown in Figures 8-2 and 8-3 indicate that the reaction suffers from limitations preventing the maximum theoretical yield from being achieved. The lower yield achieved by reactions with low catalyst concentrations can be a result of some limitation.

Plotting catalyst*time against product concentration (known as Selwyn's test) for identification inactivation of the biocatalyst over the course of the reaction has been proposed previously[165]. This plot can be used as a means for identifying rate limitations in the reaction system. If the rate of the reaction does not linearly scale with biocatalyst concentration during any part of the reaction curve, the profiles for the different biocatalyst concentrations would not superimpose on one another. Furthermore, the plot can be used for separating rate limitations that are caused by catalyst deactivation from those caused by mass transfer limitation[166].

For example, if the limitation is due to loss in stability of the biocatalyst, then the deviation would be observed in the lower catalyst concentrations. For example, for a system with product inhibition, the initial conditions would superimpose for all catalyst concentrations but as the reaction proceeds, inactivation occurs and deviations will be observed.

On the other hand, if the limitation is arising from the mass transfer limitations of oxygen, the deviations would be observed for higher catalyst concentration. This is because oxygen is required in stoichiometric amounts for the reaction. Therefore, initial rate would give an idea about oxygen limitation.

Therefore, to identify the type of limitation (for reaction system), the product concentration was normalized against enzyme concentration to obtain a product concentration against cell concentration * time plot (Figure 8-5).

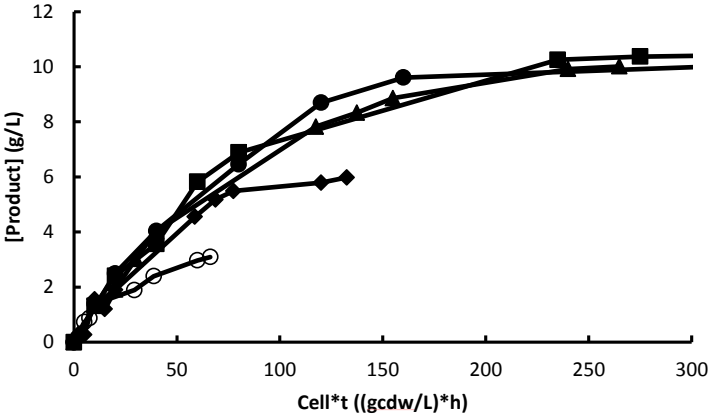


Figure 8-5: Product concentration achieved in a reaction where a substrate concentration of 14.8 g/L was converted as a function of reaction time and biocatalyst concentration. Biocatalyst concentration: (●) – 20 gcdw/L, (■) – 10 gcdw/L, (▲) – 5 gcdw/L, (♦) – 2.5gcdw/L and (○) – 1.25 gcdw/L.

In Figure 8-5, the trends for the different biocatalyst concentration overlap in the initial part of the curve, indicating that initial rate is not limiting for the catalyst concentrations tested in this case. With the current expression levels, the initial rate of biocatalysis is not seen to be limiting implying that oxygen is not limited in this reaction at the conditions tested. Since oxygen can be supplied at a rate of 100 mmol/L/h (corresponding to 14.8 g/L/h) and the current reaction rate for a reaction with 14 g/L of substrate concentration and 20 gcdw/L is 2.11 g/L/h, there is scope for increasing the rate at least by five times before oxygen becomes a limiting factor. A linear increase in reaction rate with cell concentration was shown also in Chapter 7 indicating that the oxygen is not the limiting factor. Similar plots were made for the reactions with lower substrate concentrations and verified that reaction is not limited by rate (see Appendix VII).

Figure 8-5 shows that the final product concentration achieved at biocatalyst concentrations of 1.25 gcdw/L and 2.5 gcdw/L are lower than the maximum possible product concentration. This indicates that the reaction suffers from loss of biocatalyst stability.

8.3.3 Product concentration limit

The final product concentration achieved at a particular biocatalyst concentration was plotted (Figure 8-6) to identify the maximum product concentration achievable in the system. From Figure 8-6, it can be seen that the product concentration increases linearly with increase in biocatalyst concentration until a certain

point (until biocatalyst concentration 10 gdcw/L) after which there appears to be a levelling off. The linear part of this curve depicts the total turnover number (TTN) for the biocatalyst at its current performance.

From the substrate feeding profiles, it can be seen that at a catalyst concentration of 20 gcdw/L all of the substrate was converted and therefore, this trend can be because of the unavailability of the substrate and the region has been marked as “substrate limited”. Substrate limitation could in principle occur when the substrate is volatile. However, for the current case study, the substrate conversion profiles indicate the presence of the substrate and hence volatility can be neglected.

The linear region where the product concentration increases with increase in biocatalyst concentration can when there is a biocatalyst limitation and is represented by the term “biocatalyst limited” (Figure 8-6). As an extension, if only the linear region of the plot is considered, then the area above the curve is biocatalyst limited (i.e. the entire potential of the biocatalyst has been used) and when the system is operating in the area below, it is limited by the availability of the substrate.

The biocatalyst limitation can occur either due to inactivation of the biocatalyst due to product toxicity or due to loss in stability of the biocatalyst over time at reaction conditions (i.e. interaction of components from the reaction mixture that is not related to the substrate or the product). From Figure 7-10 it can be concluded that the biocatalyst is stable at the reaction conditions for 52 hours. Therefore, loss of activity within that time should be linked to either the substrate or product toxicity. Substrate toxicity was tested for by running a fed-batch reaction and comparing it to a batch (Figure 7-12). This test indicates that the reaction is not limited by substrate toxicity. However, from Figure 7-13 it can be seen that the biocatalyst is severely affected by product toxicity and toxicity is a function of duration of exposure and the product concentration. Therefore, the final product concentration attained at low cell concentrations represented in Figure 8-6 is a function of product toxicity. In a Bayer Villiger Monooxygenase (BVMO) system, it was seen that the product concentration increased linearly with product concentration and levelled off when the toxicity became predominant[164]. Such a trend can be expected in the target system at higher biocatalyst concentrations (≥ 15 gcdw/L).

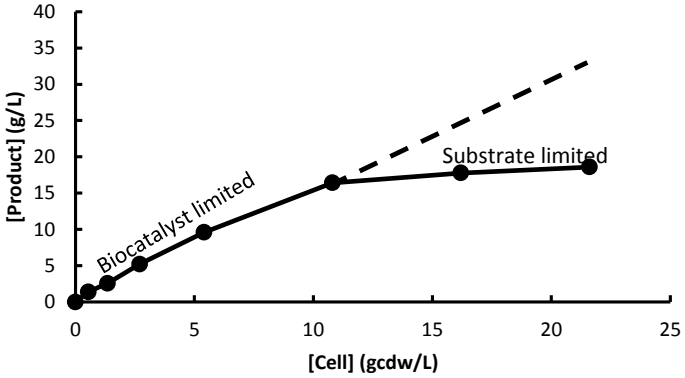


Figure 8-6: Product concentration as a function of biocatalyst concentration.

8.3.4 Implication of the results

The process metrics i.e. product titre, biocatalyst yield and maximum rate are all affected by the biocatalyst concentration. Therefore, these metrics were plotted against the cell concentration to obtain Figure 8-7. Biocatalyst yield is obtained by dividing the product concentration to the catalyst concentration.

The initial rate is obtained for a reaction with 14 g/L substrate concentration. It can be seen that the initial rate can be increased by approx. 7.4 times before the system reaches an oxygen supply limit. Improving the expression under this limit would in turn improve the product concentration and consequently the biocatalyst yield until product toxicity becomes limiting. The initial rate of the reaction can be increased by either increasing the amount of enzyme in a gram of cell or increasing the specific activity of the enzyme. However, the SDS experiment represented in Appendix III indicates that protein was not detected. It can be inferred that the protein per g of cell is very low and is possibly the parameter that can be improved.

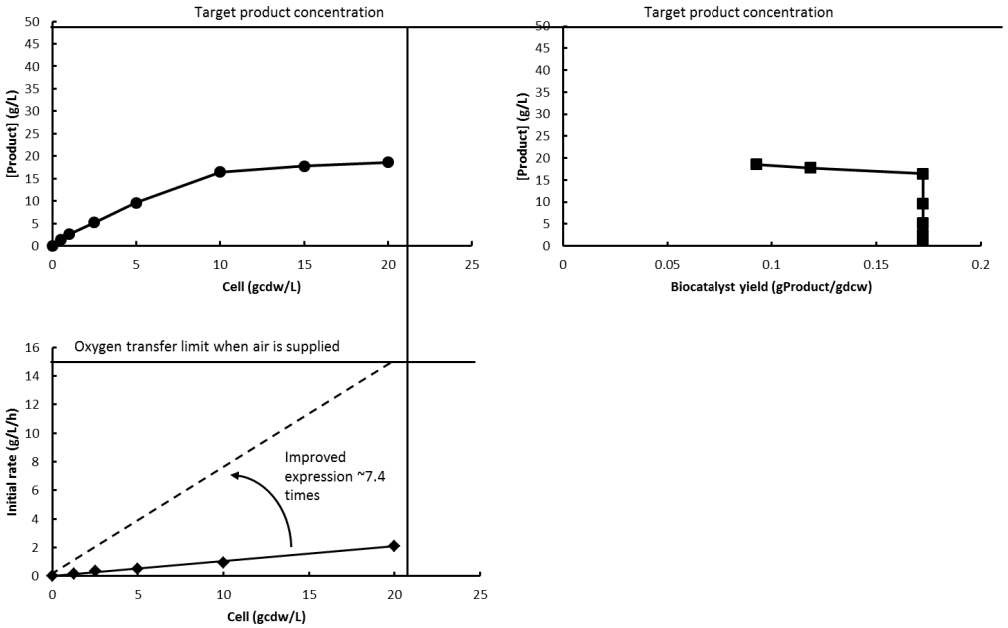


Figure 8-7: Representation of the targets and limitations for the reaction system. (●) represents the product concentration as a function of cell concentration; (♦) represents the maximum rate of the reaction as a function of cell concentration and (■) represents a plot of one economic target (product concentration) against another (biocatalyst yield).

An improvement in the initial rate would in turn change the profile of product produced per mass of cell. Since product toxicity is established as a time and concentration based phenomena (Chapter 7), an increased reaction rate would lead to an increase in product concentration achievable at a certain biocatalyst concentration. Therefore a change in the expression level would not only cause a change in the product concentration but also a change in the biocatalyst yield obtained. Similarly, improvement of product tolerance or adopting ISPR would lead to an improvement in the product concentration attained by a particular cell concentration. Hence, both the approaches have the potential for bringing the process closer to the economic targets. In order to choose one method over another, the benefits attained in the economic targets can be assessed. This is done in the following sections.

8.3.5 Effect of adopting a successful ISPR

As mentioned earlier, product toxicity can either be overcome by employing a robust ISPR technique or by protein engineering. Ideally, one of the two strategies may be able to overcome the issue of product inhibition, but a combination of these techniques is often necessary. Although ISPR is a very valuable tool, it is often difficult to employ. In order to assess the improvement achieved in the system by removing

product inhibition, data set from a successfully implemented ISPR technique is necessary. However, since this data is unavailable for this system, the improvement is estimated based on several assumptions.

To make a best-case estimate for the potential of a process implementing ISPR, the following assumptions have been made - (i) the duration of a batch is assumed to be 24 h (ii) the biocatalyst is stable during this period (chapter 5) (iii) feeding of substrate is employed such that the substrate concentration is a constant over a period of time (iv) feeding of the substrate does not cause dilution (v) an ideal ISPR is employed which removes all the product as and when it is formed and (vi) as a consequence of iii-v, the initial rate is the rate throughout the duration of the batch.

Improvement in product concentration from employing a successful ISPR

Based on the above-mentioned assumptions, plots of product concentration achieved at the end of 24 h and biocatalyst yield against cell concentration are represented in Figure 8-8 for both the base case and the case where ISPR is employed. Biocatalyst yield is calculated by taking the ratio of the product concentration to the biocatalyst concentration. The target biocatalyst yield as represented in Table 8-1 is 10-35 KgP/Kgcdw. However, since the biocatalyst is a speciality biocatalyst and is in development stage, a target of 10 gP/gcdw was chosen as the required biocatalyst yield.

In order to calculate the product concentration when ISPR has been successfully implemented, the initial rate achieved at different cell concentrations (in the base case) was multiplied by the duration of the batch (24 h).

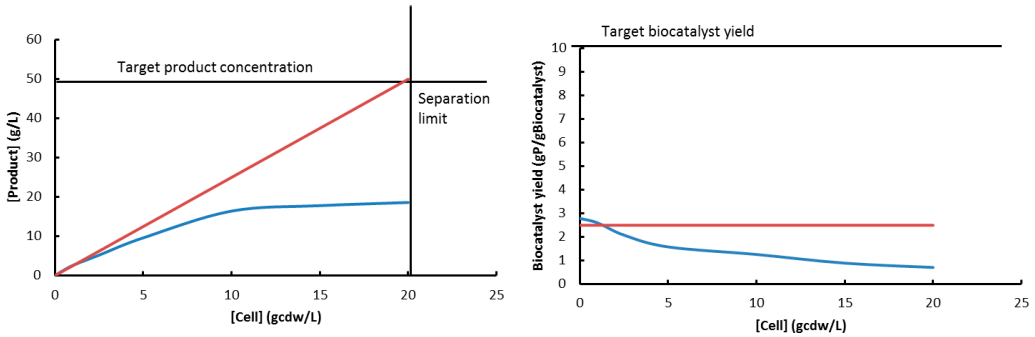


Figure 8-8: Effect of employing successful ISPR on the final product titre and biocatalyst yield. Blue line indicates the current scenario for the reaction system while the red line indicates the scenario when ISPR is adopted.

From Figure 8-8, it can be inferred that the product concentration can be increased by adopting ISPR and depending on the duration for which the initial rate can be maintained, the final product titre achieved can be varied. However, the biocatalyst yield is still 2-folds lower than the target biocatalyst yield. Therefore an improvement on the biocatalyst yield is required to make the cost contribution from the biocatalyst lower.

8.3.6 Effect of improving protein expression

The expression of an enzyme can be increased by either increasing the specific activity or the amount of protein per unit cell mass. While increasing the specific activity involves changing the protein structure, the increase in protein can be achieved by increasing the copy number. Both these strategies will influence the product concentration achieved in the end in different ways. Increase in protein concentration can only be done so long as inclusion bodies (yielding unusable protein) are not formed. Increasing the specific activity on the other hand increases the number of catalytic cycle per unit enzyme and therefore might lead to a lower stability of the catalyst. For further analysis, on product concentration and biocatalyst yield, a region where oxygen limitation does not exist has been chosen. At regions where oxygen becomes limiting, oxygen has to be supplied externally. In turn, this would cause changes in the biocatalytic reaction (for example changes in stability of the biocatalyst).

In the following discussion, it is noteworthy that an assumption has been made that the expression has been improved 5-folds and all the protein obtained is active.

However, a 5-fold improvement in expression will not translate to an improvement in the reaction rate by 5 folds because the system will likely run into mass transfer limitations when the expression is improved (as seen in Chapter 7). Only 10% of the substrate was observed within the cell, therefore when the conversion rate is increased, the mass transfer of the substrate across the membrane would become limiting. The mass transfer issues can be overcome by operating with lyophilised cells and the analysis described further will be carried out with lyophilised cells to avoid mass transfer issues.

An additional uncertainty that arises with improved expression is because of the nature of product toxicity (i.e. product toxicity is a function of duration of exposure and the product concentration). Partitioning of the product was found to be such that 50% of the product was trapped within the cells (or associated with the cell membrane). Hence, when lyophilised cells with its expression improved are used, it is likely possible that the product concentration in the microenvironment of the biocatalyst is lower, consequently there will be an improvement observed in the stability profiles.

However if we assume the use of lyophilised cells which exhibit the same stability profiles, the product concentration achieved in the linear region observed in Figure 8-6 can be used for predicting the product concentration. For an improvement in expression by 5 folds, the product concentration obtained from 1 gdcw/L would be equivalent to that obtained with a biocatalyst concentration of 5 gdcw/L in the base case. Therefore, improvement in expression would potentially lead to the use of a lower cell concentration, consequently improving the biocatalyst yield.

8.3.7 Improvement on process metrics achieved

It is a common practice to represent the biocatalyst yield and the product concentration to depict the current scenario of the process[160]. In this case however, it can be seen that the biocatalyst yield varies with the cell concentration in both the base case as well as when the expression is improved. The product concentration achieved in both cases is also dependent on the catalyst concentration. In order to compare the improvements offered by each strategy, the catalyst concentration has been fixed based on the target biocatalyst yield (10 gP/gbiocatalyst) and the product concentration (50 g/L) to 5 gdcw/L.

In Figure 8-8, it can be seen that the successful implementation of the ISPR leads the increase of the product concentration and biocatalyst yield from 2 g/g and 10 g/L to 2.5 g/g and 12.5 g/L. The base case values and the improved ISPR have been plotted in Figure 8-9.

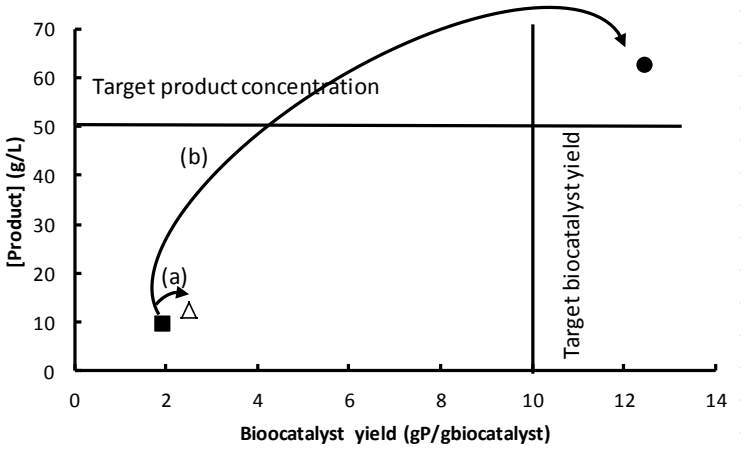


Figure 8-9: Representation of the effect of adopting ISPR and/or improved expression on the economic targets. (■) Base case at a catalyst concentration of 5 gcdw/L, (a) Effect of improving product tolerance through ISPR (b) represents the effect of improving both the product tolerance and the expression levels in a cell-free extract.

From the plot (Figure 8-9), it can be observed that the extent of improvement in biocatalyst yield and product titre achieved varies with the approach taken. Improving product tolerance seems to increase the product titre, albeit very little. But on adopting an improvement of both these strategies, the reaction system reaches the required targets.

Of the two targets, it could be argued that the product concentration is seemingly the most important one as the cost and the ease of the downstream processing for the product is directly affected by the product concentration. As a result, it is desired that a high product titre is obtained from a reaction. Consequently, a stable biocatalyst that can handle high product concentrations should be available for a target reaction.

8.4 Conclusion

A systematic analysis of the limitations for the target system has been established. The analysis presented can be extended to other oxidase based biocatalytic processes to identify process limitations. In order to identify the key process limitation however, knowledge on the cost benefits is required to be coupled with the above presented methodology. In this particular case, both the biocatalyst tolerance to the product and the biocatalyst expression has to be improved to make the process economically viable.

9. Biocatalyst washing

In order to save on the cost-contribution from a biocatalyst, it is desired that the biocatalyst is employed in the crudest form possible and it is for this reason that the rational choice of biocatalyst would be a whole-cell (when cells cannot be used in growing state). When using resting cells for biocatalysis, it is important to take care in the washing steps downstream of cell harvest. This chapter describes the technique involved in handling of the biocatalyst downstream of fermentation.

9.1 Introduction

It is desired that the biocatalyst is employed in the crudest form possible and it is for this reason that the rational choice of biocatalyst would be a whole-cell[167]. Whole-cell biocatalysts are used in cases where there is a need for co-factor regeneration. Additionally, the enzymes in whole-cells tend to be more stable because they are surrounded by their natural environment[17,168].

There are two common modes of operating a whole-cell based biocatalytic reaction (Figure 9-1). The former deals with combining the fermentation and the biocatalysis into a single step and the latter where the fermentation is separate from the biocatalysis. Host cell proteases are produced during the fermentation process which can break down the protein of interest[169]. By separating the residual fermentation media prior to biocatalysis, extracellular proteases produced by the cells can be removed. Also, when the biocatalysis is operated separate to the fermentation, more flexibility in terms of operational conditions can be achieved for these independent processes. Examples of the flexibility achieved include changing catalyst concentration that goes into biocatalysis, use of different temperature and pH criteria in fermentation and biocatalysis for achieving optimum concentrations in each of these individual steps. Therefore it is of significant advantage to de-couple the fermentation from the biocatalysis. Several biocatalytic processes employ this strategy. However, the handling of cells downstream of fermentation is quite different. Some investigations report the direct use of the cells after harvesting in the biocatalytic process in quesiton[153,157,170], cells have been washed in buffer prior to use in other cases[171-175]. The reason for the practice of washing cells prior to biocatalysis is often not documented and we believe that it is intuitively done to remove residual media components and metabolites produced during fermentation.

In this chapter, the differences in activity of the whole-cell biocatalyst before and after washing for the target reaction system are presented.

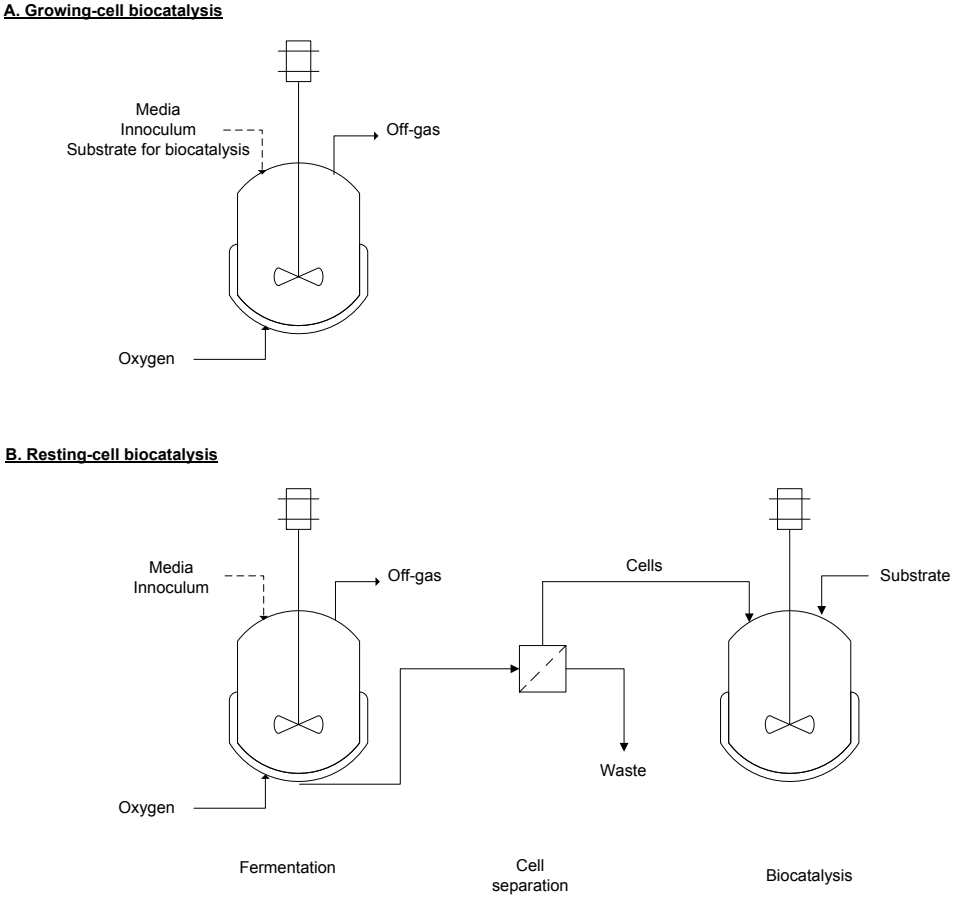


Figure 9-1: Modes of operation of whole-cell biocatalysis. (A) Growing-cell biocatalysis (B) Resting-cell biocatalysis.

9.2 Materials and Methods

All chemicals unless specified were purchased at Sigma Aldrich (Steinhiem, Germany) and used as purchased. The solvents were GC grade while the salts were analytical grade.

9.2.1 LB broth and plates

LB broth was made with 10 g/L tryptone (Nordic biolabs AB, Täby, Sweden), 5 g/L yeast extract (Nordic biolabs AB, Täby, Sweden) and 10 g/L sodium hydroxide. To this, 2% (w/v) agar was added for making plates. The broth was autoclaved and ampicillin (filter-sterilized) was added to the broth (100 µg ampicillin/mL media) prior to use and the agar just prior to plating (100 µg ampicillin/mL media).

9.2.2 TB broth

TB broth was prepared by mixing 12 g/L tryptone (Nordic biolabs AB, Täby, Sweden), 24 g/L yeast extract (Nordic biolabs AB, Täby, Sweden), 4 mL/L glycerol, 12.54 g/L potassium mono-hydrogen phosphate and 2.31 g/ potassium di-hydrogen phosphate.

9.2.3 Biocatalyst production

Pre-culture

Cells were streaked from a glycerol stock onto LB plates containing ampicillin. The plate was incubated at 37 ^{o}C overnight. 5 mL of LB broth containing 100 µg/mL of ampicillin was inoculated with a colony from the LB-Amp plates. The culture tube was incubated at 30 ^{o}C in a shaker at 150 rpm for about 4 hours (OD_{600} 0.6-1.0).

Fermentation

All fermentations were carried out in un-baffled shake flasks. To 100 mL of media containing ampicillin, 1 mL of pre-culture was added (1% inoculum). The flask was incubated at 30 ^{o}C, 150 rpm. Cells were harvested after 20 h of growth by centrifugation at 4000 rpm, 20 min and 4 ^{o}C. Fermentation was carried out prior to each biocatalytic reaction.

9.2.4 Biocatalyst washing

Biocatalyst obtained from TB and LB media by centrifugation was weighed and re-suspended with 100 mM phosphate buffer containing mono and di-basic potassium salts at pH 7.6 (25 ^{o}C). Subsequently, the cells were centrifuged again and the supernatant discarded. The pellet was re-suspended in phosphate buffer to get a concentration of ~50 gcdw/L. This concentrate was diluted to perform the biotransformations.

9.2.5 Biocatalysis

Biocatalytic reactions with whole-cells obtained from fermentation carried out in LB and TB media were carried out in baffled shake-flasks with 20 mL working volume (substrate and biocatalyst suspended in 100 mM phosphate buffer, pH 7.6) incubated at 37 ^{o}C and 150 rpm. The substrate, aza-bicyclo-octane HCl was procured from AK Scientific, (Union City, CA, USA). The sampling frequency and duration varied depending on the experiment. Samples for gas chromatography were prepared and analysed as described in Chapter 4[145].

9.2.6 Sonication

In order to obtain crude cell extract from cells grown in TB media, the cells were harvested by centrifugation and re-suspended in phosphate buffer to get a final concentration of ~500 gcww/L. 5 mL of this solution was further diluted to 10 mL with phosphate buffer. The cell suspension was then sonicated at 50% amplitude and 0.5 s cycles for 7 min. This suspension was directly used for biocatalysis.

9.2.7 Effect of glycerol on the biocatalysis

Biocatalysis was carried out using whole-cell biocatalyst obtained from LB media. The cells were harvested after shake-flask fermentation as explained previously. Different quantities of glycerol were added to baffled-shake flasks with a total working volume of 20 mL of reaction mixture such that a final concentration of glycerol was 0.025, 0.05, 0.1% (v/v). Samples were taken at 0, 15, 30, 45 min., 1, 2, 4, 6, 24 hours and analysed in GC. Initial rates were calculated.

Biocatalysis was performed before and after washing and the conversion of the substrate compared.

9.3 Results and Discussion

Several whole-cell oxidation reactions have been documented which include oxidases, oxygenases and monooxygenases. The drive for using a whole-cell for biocatalysis is either the need for co-factor regeneration, a need for a redox partner or the need to maintain the stability of the biocatalyst[176]. A major part of the cost of biocatalysis depends on the catalyst cost[167]. This in turn is dependent on the fermentation yield and the amount of active protein within the cell. For the monoamine oxidase system chosen for this reaction, the enzyme is soluble[51] and the protein is constitutive (i.e. the enzyme is produced along with cell growth and does not need to be induced, Appendix III). Consequently, the protein content per g dry cell weight is low. Thus, in order to ensure enough biocatalyst is present in the system, it is

desired to use a large quantity of cells containing MAO for biocatalysis. As a result, it is necessary to produce high concentrations of cells during fermentation. Catalyst concentration can then be adjusted by concentration or dilution to fit the need of the biocatalysis.

9.3.1 Biocatalysis in LB and TB media

In order to obtain higher biomass concentrations from the fermentation, it was desired that the media be changed to include a carbon source and eventually move to a fed-batch mode of fermentation. To assess the effect of growing the cells in the presence of a carbon source, preliminary shake-flask fermentation of cells grown in TB media with glycerol as a carbon source was performed. The switch from LB media to TB media caused an increase in cell mass obtained (in shake-flasks) from ~1 gdcw/L for cells grown in LB to ~1.5 gcdw/L for cells grown in TB. Following cell harvest by centrifugation, the cells were suspended in phosphate buffer to obtain a biocatalyst concentration of ~5 gdcw/L (i.e concentrated) and the biocatalysis was followed as substrate conversion (substrate conversion is traced instead of product formation to account for the trimerization of the product as discussed in Chapter 6) and plotted (Figure 9-2). It can be seen from Figure 9-2 that conversion is affected when the biocatalyst was grown in TB media. Both the final conversion (achieved at the end of 24 h) and the rate of substrate conversion are affected (lower for biocatalysis with cells grown in TB media).

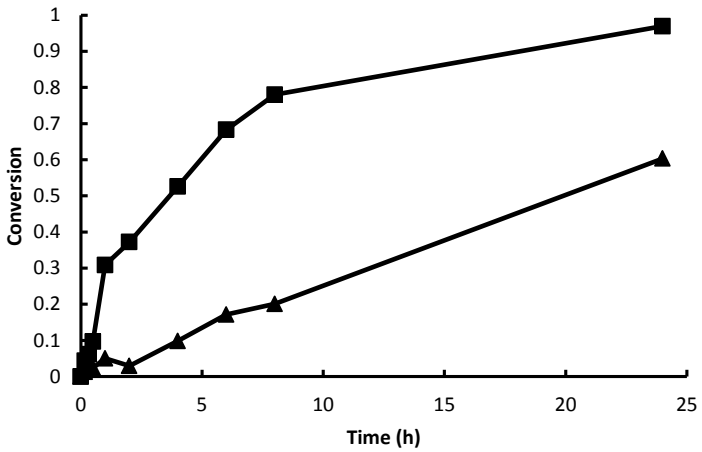


Figure 9-2: Conversion profile of biocatalysis carried out by cells grown in LB media (■) and TB media (▲).Cell concentration of 5 gcdw/L and substrate concentration of 2.96 g/L.

It was speculated that the results could arise from two separate phenomena (i) the cells when grown on TB or semi-defined media have a defined cell membrane causing a mass transfer limitation and (ii) media components cause inhibition to the biocatalyst.

In a study with yeast cells, it was seen that when the cells were grown in nitrogen limited conditions, the cell membrane was more porous than when it was limited by carbon source (glucose)[177]. It was therefore speculated that membrane porosity and consequently the mass transfer across the membrane could affect the biocatalysis. In order to test mass transfer limitations due to cell membranes, the cells were sonicated and used for biocatalysis (Figure 9-3). The trend for conversion for cells grown in TB media was similar to the cells grown in LB indicating that the mass transfer limitations could arise from a more developed membrane. However, the rate was a little lower than when cells were grown in TB media although the final conversion achieved was the same (It is noteworthy that the catalyst concentration is the same in both the biocatalytic reactions). It was speculated that the media components could cause inhibition which leads to such a phenomena and this is discussed further.

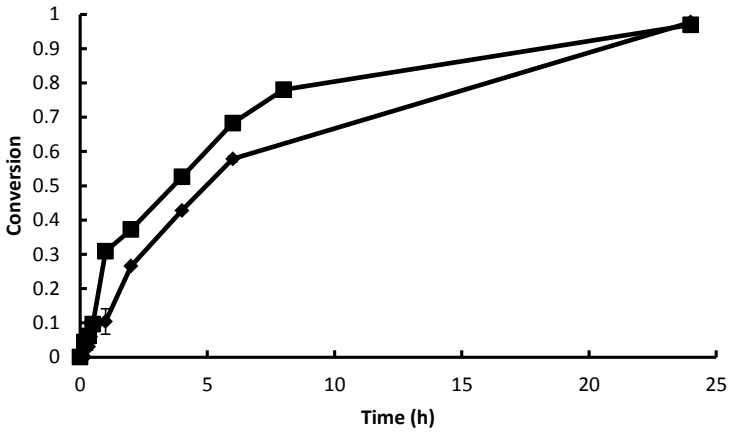


Figure 9-3: Conversion of substrate by cells grown on LB media (■) and crude extract on TB media (♦). Duplicates were made for the reaction with crude extract and the error bars drawn are indicating the standard error between the duplicates.

9.3.2 Test for media component inhibition

In order to test for inhibition from media components, the cells were washed with phosphate buffer to remove any media components from the cell suspension. The reaction profiles indicate that the washed TB cells perform as good as the LB cells that were also washed with phosphate buffer (Figure 9-4). This test further indicated that media components from fermentation broth might be influencing the biocatalysis

reaction. It should be noted that the washing step involves using phosphate buffer to re-suspend the cells after harvest by centrifugation followed by another round of centrifugation and re-suspension before the cells are used for biocatalysis. This procedure is believed to cause some damage to the cells (through lysis of the cell membrane) causing some of the biocatalyst to be lost during the centrifugation step). Therefore it is necessary to handle the catalyst from LB- and TB-grown cells in the same way to avoid differences that arise from the loss of biocatalyst. Washing the whole-cells after fermentation seems to improve the conversion for cells grown in TB media (see Figure 9-2 and Figure 9-4). Therefore, the effect of residual media components on biocatalysis was tested by adding media component that is different between the two media (LB and TB) back to the biocatalytic reaction mixture.

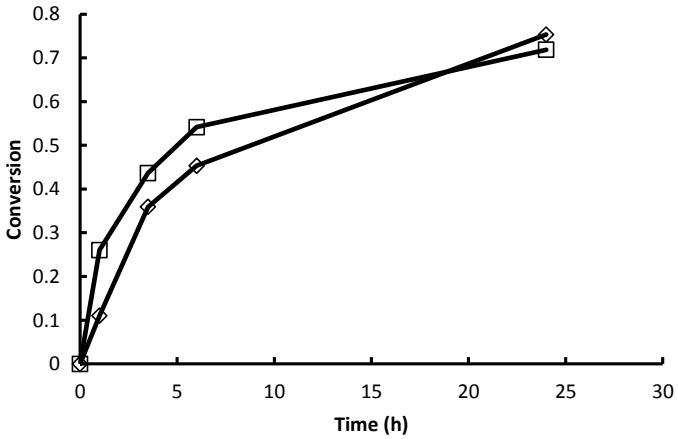


Figure 9-4: Comparison of substrate conversion profiles from cells grown in LB (□) and TB (◊) media that were washed prior to biocatalysis.

The primary difference between LB and TB media is that the TB media has an additional carbon source (glycerol). The cells were grown by shake-flask fermentation prior to biocatalysis. Hence it was speculated that there might be residual glycerol in the reaction media that can cause a negative impact to the reaction. Trace amount of glycerol was added to the reaction mixture and the reaction progress was measured. Glycerol is expected to be consumed by the cells during fermentation, however since the cells were grown in shake-flasks, complete utilization of glycerol may not occur, therefore, only trace quantities of glycerol were added to the reaction mixture. The overall reaction trends were similar and all the reactions reached complete conversion (Figure 9-5). However, there was a significant difference in the initial rate was observed when the glycerol concentration was increased (Figure 9-6). This indicates that media

components (glycerol in this case) can affect biotransformation reactions and it is important to wash cells prior to catalysis.

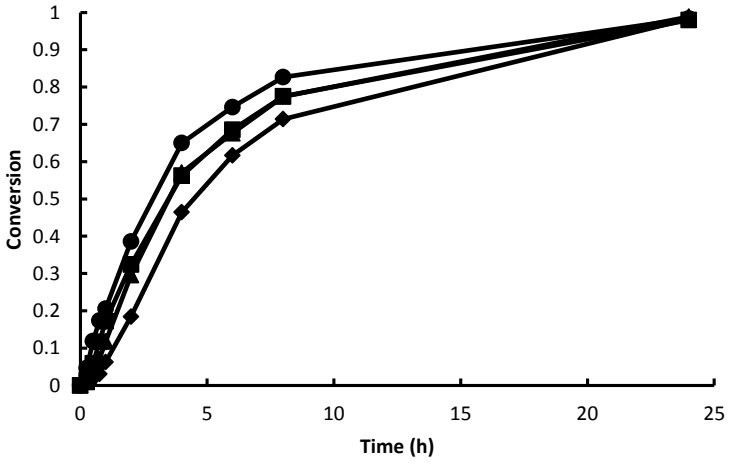


Figure 9-5: Biocatalysis in the presence of glycerol. Different concentrations (●) – 0%, (■) – 0.025%, (▲) – 0.05% and (♦) - 0.1% of glycerol were assessed.

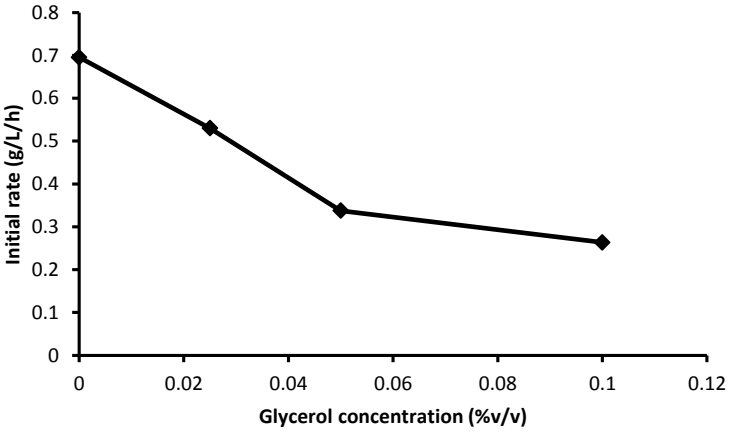


Figure 9-6: Effect of glycerol on the initial rate of the biocatalysis.

9.3.3 Implications for operating with whole-cell biocatalysts

Crudest possible form of enzyme (whole cells or crude extract) is desired to operate biocatalytic process as a rule of thumb to help reduce the cost contribution from the biocatalyst[178]. Whole-cell biocatalysis can be operated with either growing cells (fermentation and the biocatalysis occur simultaneously) or using resting cells (fermentation is separated from biocatalysis). Use of resting cells gives process flexibility in the sense that the ideal operating conditions for biocatalysis can be used as opposed to operating at conditions that is a compromise to both the fermentation and biocatalysis[179]. Considering a process is operated such that the fermentation is separated from the biocatalysis, we cannot simply dilute or concentrate the cells derived from the fermenter as discussed earlier. It might be necessary to wash the cells to remove residual media components and/or by-products from fermentation, which may interfere with biocatalysis[180]. A flow sheet of the process from fermentation to biocatalysis is presented in Figure 9-7. The cells obtained from fermentation are separated either by filtration or centrifugation and the cells are then washed with buffer to remove residual media components, followed by a cell separation stage. Following this the cells are suspended in buffer or water to obtain the desired biocatalyst concentration for the reaction. It should be noted that the addition of an extra step downstream of fermentation (which includes biocatalyst washing and an additional separation stage) would add to the cost contribution of the biocatalyst.

In Figure 9-7 the cell concentrations are depicted as cf, cfc, cw, cb. It is noteworthy that the first cell separation would lead to an increase in cell concentration up stream of fermentation. Following this, one can wash the biocatalyst and separate the waste from the washing step from the cells (resulting in another concentrated cell solution). Finally the cells are suspended in the reactor used for biocatalysis to obtain the concentration desirable for biocatalysis.

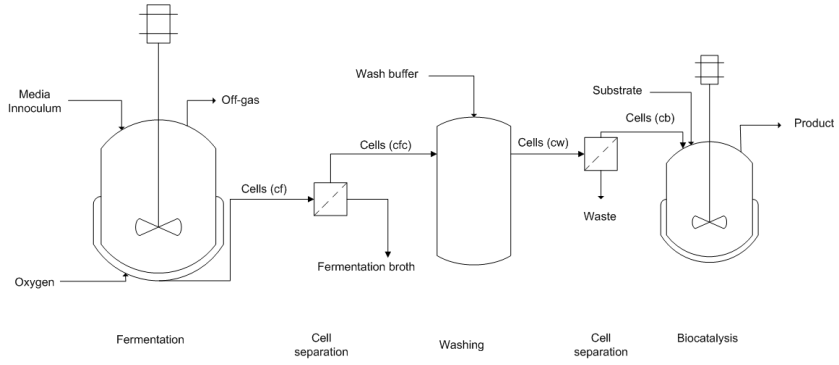


Figure 9-7: Whole-cell biocatalyst handling from fermentation to biocatalysis. Cf, cfc, cw, cb represent the biocatalyst concentrations coming from each unit operation.

9.4 Conclusions

In this study, it has been shown that washing of cells is necessary for biocatalysis especially when cells are grown in media with additional carbon source (such as TB). Although this study has been carried out using *E. coli* cells, this phenomenon of media component influencing the performance of biocatalysis might be true for other organisms as well. It is also noteworthy that washing procedure (quantity of buffer used for washing with respect to the biomass produced) needs to be assessed and could vary dependent on the cells and the ease of separation of the residual media components. Also, it is important to consider the necessity of intact cell membranes for the biocatalytic process. Introducing a washing step would cause changes in cell membrane integrity which may affect the biocatalysis. Finally, the effect of the media components on the biocatalysis could be detrimental and motivates the need for de-coupling fermentation from the biocatalysis.

Part V

10. Discussion

Summary

In this chapter, general discussions on oxidase-based biocatalytic processes based on the case study have been described. A part of this chapter presents the work in this thesis and explains how they can be extended to other biocatalytic oxidations. Concerns from the analysis of the experimental results have been raised whenever needed and forms a basis for future perspectives, which will be listed in Chapter 12.

10.1 Process development

The monoamine oxidase case study in part was dedicated to process development. Based on the results from identification of the constraints in this system through *in silico* analysis and further from experimental validation, suggestions for improvement to make the process economically feasible have been identified.

10.1.1 Methodology for process development of oxidases

Biocatalytic reactions have two major considerations that need to be taken into account for process development. The first is what a biocatalyst can provide (and what its limitations are) and secondly how process engineering can assist in improving the process. Initial work on process development has focussed on fitting the process to the biocatalyst. However, the field of protein engineering has seen tremendous improvement making it possible to fit the enzyme to the limitations of the process[120,181-183]. However, certain limitations such as volatility of the substrate(s) and product(s) or thermodynamic equilibrium limitations of the reaction cannot be overcome by protein engineering and can only be overcome by process engineering or reaction engineering. Therefore, it is desired that that biocatalyst development and process development must go hand-in-hand to overcome the limitations of a particular system. A combined effect of process development has been suggested by Ramos et al[154].

In this thesis, process development was made for the case study (MAO) where most of the published results were either in a patent or in the supplementary information of a manuscript by Köhler and co-workers[57,184]. It was therefore interesting to study the process aspects and the limitations for this bio-oxidation for scale-up.

In order to get to process development, a systematic approach has been described. Firstly, a limited number of mutants (three) of the biocatalyst were available to test for the target system. The three enzymes were first used and the best mutant was selected for characterisation. The process targets for the system were set which include product titre and the biocatalyst yield.

Following this, the most important step was to make an *in silico* analysis of the limitations of the target system to guide the experimentation and thereby help in making smarter choices between the process options available for the target system. *In silico* analysis also helps identify the potential limitations of the system prior to experimentation. For example, the differences in product and substrate properties will dictate the efficiency of product removal. The efficiency of product removal in turn will affect the product concentration that is required for separation and the cost of the downstream processing.

Form the work of this thesis, a methodology for process characterisation for resting-cell, oxidase-based biocatalytic reaction can be derived and is presented in Figure 10-1.

For the current system, *in silico* analysis provided vital information about the necessity of pH control in the target system as well as indicated the potential for substrate inhibition. Oxygen requirement was found to not be limiting for the target system. The experiments conducted were in part to validate the identified limitations from the analysis followed by quantification of other expected limitations such as product toxicity to the biocatalyst. Other limitations arising from the product have been discussed in Chapter 6. Finally a process limitation analysis has been conducted in Chapter 8, which goes to identify the bottleneck of the current process which is discussed in the following section.

The methodology worked for the monoamine oxidase case study where the substrate had high solubility. In cases where substrate solubility is limited, there will be an additional limitation arising from the mass transfer of the substrate (in the second phase) to the cells. This would need to be addressed. The critical concentration of the substrate (with low solubility) will determine the necessity for substrate solubilisation efforts. If the substrate is toxic below the solubility limit, then solubilisation would not help improve the performance of the system. However, if the substrate is toxic above the solubility limit, then solubilisation of the substrate using a co-solvent would help improve the substrate mass transfer. If a co-solvent is used, then the stability of the biocatalyst in the co-solvent needs to be assessed.

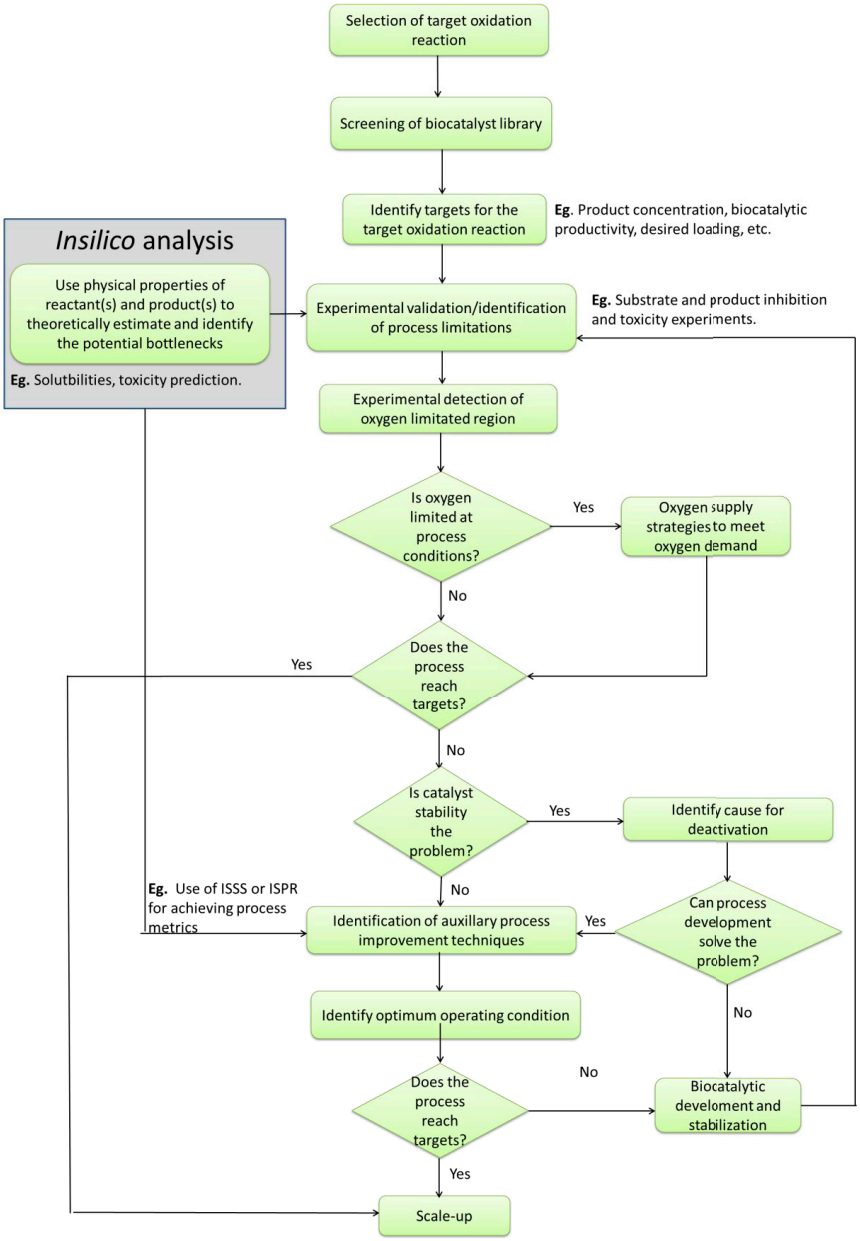


Figure 10-1: Methodology for process development for oxidases using resting cells.

To summarize, in the result presented in this thesis follow up from identification of target system down to identifying the cause for deactivation of biocatalyst. Implementation of a successful ISPR technique and scale-up remain to be achieved.

10.1.2 Process limitation analysis

For a biocatalytic process with resting cells, the cost contribution from the biocatalyst (biocatalyst yield) and the product titre are the most important economic targets that need to be fulfilled. Since the monoamine oxidase example is an oxygen requiring system, oxygen has to be supplied in sufficient quantities to cope with the maximum rate of the reaction. Therefore, maximum rate is introduced in this thesis as a constraint to the system.

To make a process limitation analysis, the effect of the catalyst concentration on the process limitation can be analysed as explained in Chapter 8. In Chapter 8, the maximum product concentration reached for a given cell concentration was studied and it was inferred that product concentration achieved was a linear function of cell concentration. It was also seen that the product toxicity limit is a function product concentration and the duration of exposure. Product toxicity can be overcome by adopting biocatalyst engineering[182] for improving product tolerance or by removing the product from the microenvironment of the biocatalyst (such as by implementation of ISPR)[155,185]. Additionally, sensitivity analysis on the effect of overcoming the process limitations on process metrics revealed that the removing product toxicity alone is not enough to make the process economically feasible. The expression of the cells also needs to be improved to make the biocatalyst cost contribution reasonable. Expression of biocatalysts can be improved by biocatalyst engineering.

When the expression levels are increased, then the potential initial rate of the reaction is also increased so long as mass transfer across the membrane is not limited. The system can go from being limited by the expression levels to limitation on transport across the membrane. However, several methods of permeabilization of the cell membrane are available and can be used[150,186]. Hence, transport across the membrane was not considered as a potential limitation to the system. Due to mass transfer limitations, it becomes advantageous to use cell-free extract or lyophilised cells.

Increase in expression, would in turn have an effect on the oxygen demand from the process (since one mol of oxygen is used for every mol of substrate converted) as long as mass transfer across the membrane is not limiting. With the current system (i.e. at the current expression limit), oxygen has been identified to not be a constraint, however, when the expression is improved, it might become a problem. Operating at high cell concentrations will in turn shift the regime to oxygen limitation. It is noteworthy that although the system might run into oxygen limitation when rates over 100 mmol/L/h are achieved, it might not be necessary to operate the reaction at rates higher than that to achieve the product titre required within the

duration of a batch. Also the effects of oxygen supply and oxygen concentration on the stability of the biocatalyst need to be examined.

Finally, substrate limitation was observed in this case study, and this has been listed as the last limitation. Various substrate supply strategies have been successfully implemented[123,187,188]. Therefore, it is not considered as a major limitation and listed as the last constraint.

To summarize, from the results in Chapter 8, it was seen that the expression of the biocatalyst need to be improved even if product toxicity is overcome in order to achieve the targets for the biocatalyst yield. Following the expression limitation, product toxicity affects the reaction system severely. Thus, an order for the process limitations specific to the monoamine oxidase case can be arrived at and is depicted in Figure 10-2. The figure has been drawn based on the assumption that there is no mass transfer limitation across the membrane.

The limitations that arise in other oxidase systems would likely have a different order of occurrence. In principle, the order of the limitations can be identified based on the improvement gained on the process metrics by overcoming the process limitations that are identified for the system.

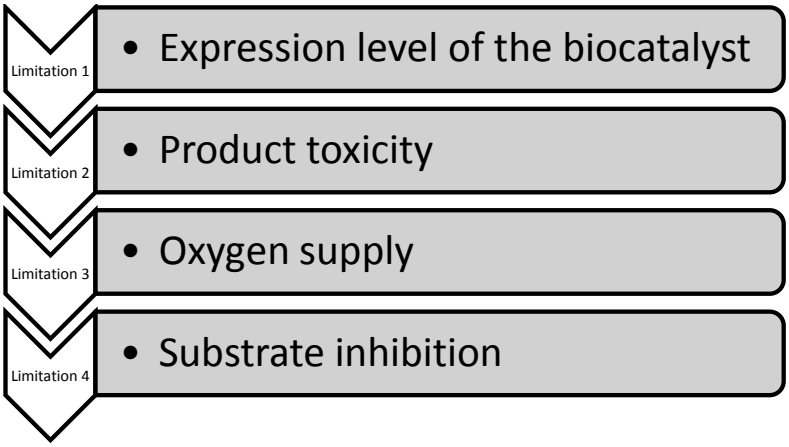


Figure 10-2: Order for process limitations of the current system assuming that the increase in expression does not cause mass transport limitation of the substrate and product from the bulk across the membrane.

10.1.3 Catalyst formulation

All most all of the experiments in this study have been carried out using whole-cells. It is desired to operate a biocatalytic process with the crudest possible enzyme in order to save on the cost contribution from the biocatalyst. Use of whole cells from fermentation saves cost on the downstream of fermentation since protein isolation steps are avoided. Also, the use of whole-cell protects the enzymes by keeping them in their natural environment and protects the target proteins from shear forces[189,190]. Whole-cells are often seen to be advantageous when co-factor regeneration is required, when a fusion-construct of a protein is desired (like in the case of P450s) or when *de novo* pathways of the cells are required for the biocatalytic process[176].

However, use of whole-cells can cause mass transport limitations of substrate and product across the cell membrane. While molecules that are nutrients for the cells are usually taken up by active transport. Transport of substrate molecules usually used for biocatalysis do not have active transport mechanisms and have to be transported by passive diffusion[191]. Therefore, cells need to be permeabilized in order to allow for transport across the membrane and prevent mass transfer limitations[192].

Furthermore, when employing whole-cells for biocatalysis, product recovery becomes more complicated due to the product being trapped inside the cell (this has also been seen in our experimental results)[193]. It was seen that 50% of the product was associated with the cell membrane which could potential imply that there is a high product concentration in the microenvironment of the enzyme. By using a lyophilised system, the product concentration at the microenvironment would be lower, reducing product toxicity. It should be noted that the catalyst recovery is made more difficult when lyophilised cells are used.

For the target system, experimental results have shown that the stability of the crude extract is as good as the whole-cells (which is often not the case in other oxidase systems, particularly amino acid oxidase). Also, there is no need for co-factor regeneration systems (since FAD is tightly bound to the enzyme). And finally, the expression levels of the enzyme are low and hence use of high catalyst concentration which causes downstream limitations in terms of product recovery.

For the current process therefore, it could be more useful to use crude extracts which opens up the possibility of loading more catalyst in the reactors and possibly ease the separation of the product from the reaction mixture. Some studies have also reported higher activities for isolated enzymes compared to whole-cells[194]. Thus, if the expression of the system was improved, it would possibly be more advantageous to shift to isolated enzyme formulation.

10.1.4 Process outlook

For the monoamine oxidase catalysed reaction, the following limitations have been identified – substrate and product inhibition, product toxicity, low expression levels and pH shift during the reaction.

The first limitation to overcome has been identified as low expression level. The consequences of improving the expression levels have been explained in the previous section. In order to avoid mass transfer limitations across the membrane, use of lyophilised cells has been suggested.

In order to lower the costs of biocatalyst production, it is desired that high cell densities are obtained from the fermentation. This necessitates the use of semi-defined media such that the fermentation is operated in a fed-batch mode. Consequently, it becomes necessary to wash the cells downstream of fermentation to remove residual media components and fermentation by-products (as seen in Chapter 9, use of high cell density media causes inhibition of the enzyme lowering its activity).

Chapter 6 indicated that a pH shift should be expected for the target reaction system. Therefore, pH control is required for the process to be successful.

In Chapter 7, it was seen that the biocatalyst was limited by product toxicity. To overcome product toxicity, ISPR using absorbent resins have been suggested. Also, the optimum pH for separation was identified to be different from the pH optima for the reaction. As a result, pH adjustment is required before and after ISPR operation.

From the above discussed constraints and assuming that the expression of the system is improved and ISPR operation using resins is successfully implemented, the process outlook from the biocatalyst production to product recovery can be depicted in Figure 10-3. First, the cells will be produced in a fed-batch mode of fermentation (to obtain high cell densities) followed by cell separation. Following this, the cells need to be washed to remove residual media components and the cells disrupted to get crude extracts. The crude enzyme would be used in a stirred tank reactor where oxygen supply, oxygen monitoring and pH control will be employed. Following the reaction vessel, an external packed bed with absorbent resins will be used for product removal. pH adjustment has to be made before ISPR to ensure that the process is operating at optimum conditions. A reverse osmosis step after the second pH adjustment is employed to prevent dilution of the reaction system.

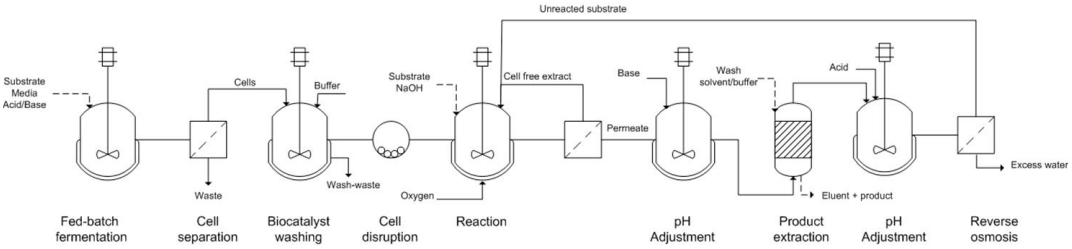


Figure 10-3: Process outlook for monoamine oxidase catalysed biocatalysis assuming that the expression of the biocatalyst has been improved.

The process can be extended to other biocatalytic processes. While the fermentation is preferably carried out in fed-batch mode, the catalyst formulation used depends on a case to case basis. For example, an extracellular protein or a whole-cell resting state based biocatalysis would not require cell disruption techniques.

For biocatalytic processes, high processing intensities are usually desired. As a consequence, it is desired to operate at high substrate concentrations. While substrate inhibition and toxicity levels can vary, they will likely be present for biocatalytic processes and therefore a fed-batch mode of operation is desired. Similarly, to help the biocatalyst cope with high product concentrations, product removal strategies have to be applied. Depending on a case to case basis, the product removal strategy adopted would vary.

10.2 Oxygen sensors as tools for oxidase-based biocatalytic processes

The work presented in Chapter 4 describes the use of oxygen sensors for measuring Kla and for obtaining substrate inhibition profiles. The sensors in extension could be used when there is a change in oxygen concentration to track the progress of the reaction as well as for cases where kinetics for oxygen requiring reactions is desired. Nevertheless, care should be taken when implementing these sensors when the oxygen is produced or consumed by a side-reaction. For example, in a biocatalytic oxidation, if catalase is present in the enzyme formulation, it is desired to quantify the activity of the catalase and account for the oxygen produced during the degradation of hydrogen peroxide. Similarly, when product is labile or is substrate is used in side-reactions, it is likely that the oxygen sensors cannot be used for quantitation of the reaction. In these cases, it is necessary to make more experiments. The implementation of such sensors in biocatalytic oxidations can nevertheless provide a valuable tool for monitoring and control of the process.

The following chapters would describe the conclusions of the thesis and some directions for future work.

11. Conclusions

Biocatalysis has moved from a time where there was a lot of scepticism around the industrial application of biocatalytic reaction to where there are over 150 industrial reactions implemented at industrial scale[17,195]. Particularly, biocatalytic oxidations have been advocated in light of high biocatalytic selectivity and specificity. This is not without sound reason. Oxidases in particular have harnessed interest for industrial applications. The potential of oxidase reactions can be seen from the industrially implemented processes that employ oxidases (amine oxidase, amino acid oxidase and glucose oxidase).

This thesis has focussed on process development for oxidase-based biocatalysis. Here, the biocatalyst was used in a whole-cell formulation (in resting mode) for catalysing the oxidation of a secondary amine to an imine (which is used as a building block for a drug to treat Hepatitis C).

The significant conclusions from the MAO catalysed reaction system have been classified and listed.

- A methodology for process development of oxidase based reaction has been arrived at in this thesis. The methodology includes the use of property prediction tools to gain process knowledge prior to experimentation. It was identified that oxygen supply will likely not be a problem for this system. While substrate inhibition and (co)product inhibition are potential limitations. The use of *in silico* analysis of the reaction system helped direct experimental work for the thesis.
- *In silico* analysis of the substrate toxicity indicated that the substrate is toxic at concentrations over 100 mM (~14.8 g/L) and was in line with experimental results.
- Following this, experimental evaluation has been adopted to quantify the limitations that are present in the system. From the experimental evaluations co-product inhibition from the hydrogen peroxide was identified to be unproblematic due to the presence of catalase to cope. Product toxicity was identified as a potential limitation. Product toxicity has been established as a phenomenon that is time and catalyst concentration dependent for example exposure to approximately 1 g/L of product for 24 hours caused the activity of the biocatalyst to decrease by about 50%.
- pH control was established as a critical process parameter that needs to be closely controlled. The biocatalyst lost ~50% of its activity with a pH shift of 1 unit.
- The product produced in the target reaction system was identified to co-exist as a trimer which was not detectable by GC.

- Partitioning of the substrate and product within the cell and the reaction media is highly critical for whole-cell biocatalytic systems. About 10% of the substrate is within the cells while 50% of the product is trapped within the cells. Therefore, the product trapped in the cell should be considered for implementation of ISPR.
- When fermentation is carried out for high cell-density cultures, it is of critical importance to wash the cells with buffer to remove residual media components that hamper the activity of the biocatalyst.
- For the target reaction system, product toxicity and level of expression were identified as central hurdles that prevent the reaction from scale-up. Evaluation of the bottleneck revealed that while improvement in product tolerance would enable the target for product titre to be reached, it is imperative to improve the expression levels of the biocatalyst to reach the target biocatalyst yield. A combination of improvement of the expression of the biocatalyst in combination with the product tolerance is important for reaching the targets for the reaction.
- Oxygen sensors have been used as a monitoring tool in the presence of solvents. The sensors have been shown to work in the presence of solvents are a highly versatile tool for online monitoring. The sensors can also be used as a screening tool.

12. Future Perspectives

Based on the work performed in this thesis and some directions for future work are proposed. Furthermore, further development of the biocatalysts or the tools that are available for biocatalytic reactions can have a significant impact on the perspective of operating oxidase-based reactions. Some of these improvements required are presented in this chapter.

12.1 Future work specific to case study

Development of a feeding strategy – In this study, to identify the potential bottlenecks to the process, substrate was fed after a certain amount of time. However, this is not an efficient feeding strategy. If the substrate is fed at the rate at which it is converted, there is a potential of improving the product titre in the end (as the product toxicity limit is also dependent on the duration of exposure).

Catalyst development – During the bottleneck evaluation, it was seen that there is a need to improve the expression levels of the biocatalyst in order to operate the process within the cost contribution limit from the biocatalyst. Therefore, the expression of the biocatalyst should be improved. Since oxidases can operate outside the cell with remarkable stability, it could be an option to make the protein extracellular using a different expression system. This would allow for overexpression as well as easy recovery of the protein.

Development of a method to track the trimer form of the product – One of the most important parts of process development is to keep track of the mass balances in the system. One of the limitations in the present system is that the detection method cannot identify the product trimer. As a result, it was never possible to verify if all the substrate was being converted into the product. Therefore, it is important to develop a method to quantify both forms of the product molecules. LC-MS could be a powerful tool for such an analysis.

Overcome product toxicity – An attempt of ISPR using absorptive resins has been made in this thesis. While in principle this should work, but because most of the product (50%) was still trapped inside the cell, implementation of ISPR was difficult. For this reason, it is necessary to test the ISPR method suggested in this thesis or develop a new method using cell-free extracts to overcome the need for transport of the product across the cell membrane. Alternatively, the cells can be modified to be tolerant to high product concentrations.

Reactor engineering – Oxidases are enzyme that require oxygen supply. Some of these experiments for characterization have been carried out in shake-flasks and there is no difference in the biocatalysis profile between the shake-flask and the reactor. However, this is only true because of the low expression levels in the current system. On improving the expression, the supply of oxygen will become important and the use of reactors where air is sparged will become a necessity to harness the full capacity of the biocatalyst.

Stability – This thesis has only assessed the stability of the enzyme when there is no reaction being carried out by it (i.e. storage stability). However, stability under reaction conditions has not been assessed. This could be done by operating the system under a continuous system and tracing the conversion loss over a period of time.

Methodology development – A methodology for process development has been proposed in this thesis. However, the methodology does not include the cases where the substrate has low solubility. Also, the requirement of the cells metabolic activity has not been discussed or included. While solubility issues can occur in oxidases system, the requirement of cell's metabolic activity is more common for other oxygen requiring systems (e.g. oxygenases).

Economic evaluation and scale-up – The target monoamine oxidase system discussed in this thesis has not been scaled-up because of the need for improvement in the expression and product tolerance required to make the process economically viable. However, when these are circumvented, economic evaluation and scale-up become the apparent next step for the process.

Furthermore, some general future work can be described for oxygen-requiring biocatalytic processes.

Property prediction tools – While there are some property prediction tools available for academic use, they operate under certain assumptions (for example, solubility data available is usually presented in water at 25 ^{o}C) which may not be relevant under experimental conditions (where the solubility measurements vary due to presence of salts and the temperature is usually higher). Therefore, there is a need to develop property prediction tools which will make the *in silico* analysis more robust and consequently reduce the effort in terms of experimentation.

Modelling – It is desirable to have models for the prediction of the biocatalytic oxidation. By developing such a model (including kinetics of the enzyme) and by including the entire process (i.e. substrate supply and product removal), the effect of implementing these technologies can be identified, and would be a valuable tool.

Development of oxygen supply technologies – Since oxidases are highly efficient enzymes that require oxygen for catalysis, it is necessary to develop oxygen supply strategies that are effective. Currently, oxygen supply is done with air at the rate of 100 mmol/L/h but depending on the choice of oxidase, higher oxygen concentrations maybe desired to keep up with the oxygen demand. Hence development of new oxygen supply technologies could be a useful tool for oxygen requiring biocatalysis. It should be kept in mind that the oxygen concentration could alter the stability of the biocatalysts and will need to be evaluated.

Sensor development – The solvent-resistant oxygen sensors have been shown to be excellent tools for monitoring of oxygen requiring reactions that need to operate in the presence of solvents. However, the life-time of the sensor is currently short and need to be re-calibrated often. This is a disadvantage in operating with these systems especially in continuous reactors. Therefore, there is still some need to improve the stability of the sensors.

Use of oxygen sensor as tools for oxygen requiring reactions – The sensors can also be used in two-phase, oxygen-requiring systems that involve the presence of an emulsion of the solvent and the aqueous phases. These reactions are often run (for example to achieve substrate supply or product removal). The sensor system discussed here has been used with a catalyst formulation that does not contain catalase. In principle, when catalase is present, they would degrade the hydrogen peroxide produced to oxygen and water and would influence the concentrations. These effects need to be considered when using the system for other operations.

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Appendices

Appendix I – Calibration curve

Calibration curve for substrate

A substrate stock of 200 mM solution was made and different volumes of the substrate solution were taken in an eppendorf tube to obtain different concentrations of the substrate as represented in Table A1. The volume of the sample was made up to 200 µL using phosphate buffer at pH 7.6. Sodium hydroxide was added to make the pH alkaline (this ensures that the substrate and product molecules are uncharged). To this 1mL of MTBE was added and the sample extracted to the MTBE phase. The solvent phase was dried with sodium sulphite and analysed in the GC. Triplicates were made and plotted (Figure A1).

Table A 1: Volumes of stock solutions used to obtain the various concentrations

Volume of stock (µL)	Vol of buffer (µL)	Vol of IS (µL)	Volume of 10M NaOH (µL)	Conc (mM)
200		100	100	200
150	40	100	100	150
80	120	100	100	80
40	160	100	100	40
30	170	100	100	30
20	180	100	100	20
10	190	100	100	10
5	195	100	100	5
2.5	197.5	100	100	2.5

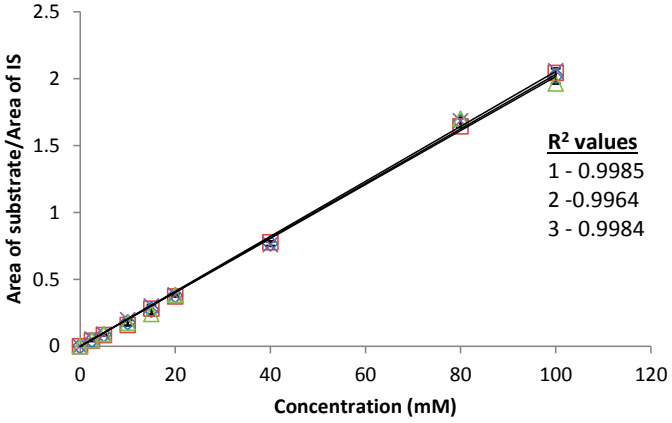

Figure A 1 : Calibration curve obtained for the substrate. Triplicates were made and the accuracy determined.

Test for instrumental error

In order to check for instrumental error in injection, multiple injections were made from the same vial and this was tested with different substrate concentrations. **The instrumental error on the injection was <1%.**

Table A 2: Multiple injections from the same vial to determine instrumental error

Replicates	1			2			3		
Concentration (mM)	Area of Substrate	Area of IS	Area Substrate/ Area IS (1)	Area of Substrate	Area of IS	Area Substrate/ Area IS (1)	Area of Substrate	Area of IS	Area Substrate/ Area IS (1)
10	135477.6	852716.7	0.15887762	136789.9	870330.3	0.15717007	106392	687819.6	0.15468015
5	54517.91	663917.3	0.082115511	66551	823563.5	0.08080858	73284.71	899059.1	0.08151267
2.5	35185.4	869735.3	0.040455298	26958.85	662819.3	0.040673	31829.99	770337.1	0.04131956

Standard curve for product

Product was synthesised at University College of London (UCL) and provided as a solid. The weight of the imine used for the standard curve is 0.02 g. The solid was first dissolved in 100 µL of concentrated HCl (6M) and then the volume was made up to 5 mL using phosphate buffer. This was used as the stock solution and the product concentrations were varied with buffer. The product was extracted to MTBE and the solvent phase was analysed.

Table A 3: Volume of stock solutions of product used for obtaining different product concentrations

Volume of stock (uL)	Vol of buffer (uL)	Vol of IS (uL)	Volume of 10M NaOH (uL)	Conc (mM)
200	0	100	10	12.21
80	120	100	10	4.885
40	160	100	10	2.443
20	180	100	10	1.221
10	190	100	10	0.611
5	195	100	10	0.305

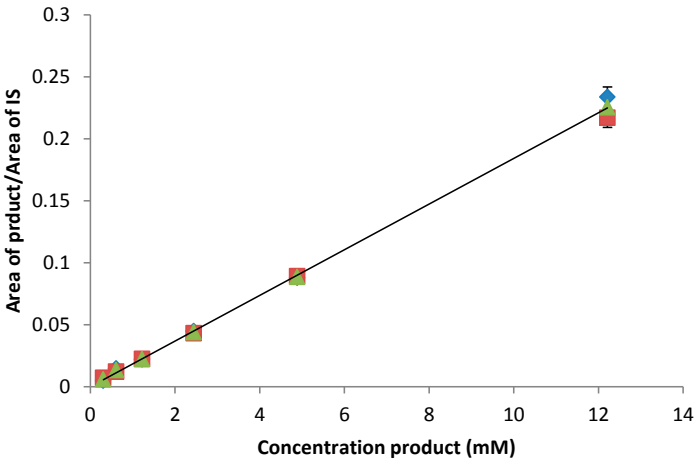

Figure A 2: Calibration curve for the product

Because the product forms a trimer which cannot be identified in the GC, this calibration curve was not generally used, but the reproducibility in the areas and concentration indicate that equilibrium in the solvent phase is constant at a given product concentration.

Appendix II – DCW measurements

A standard curve was made to convert wet cell weights obtained in fermentation and the stock solution obtained after concentration to determine the corresponding dry cell weights. Dry cell weight was measured by filtering the cells (using a vacuum pump) and drying them in a microwave (low power, 20 min). The dried filter paper was then left in the desiccator for at least an hour before the weight was measured.

Fermentation yield

In order to assess the fermentation yield in LB media, cells were grown in LB media and samples were taken at regular intervals. The OD and the dry cell weights were measured and plotted (Figure A 3).

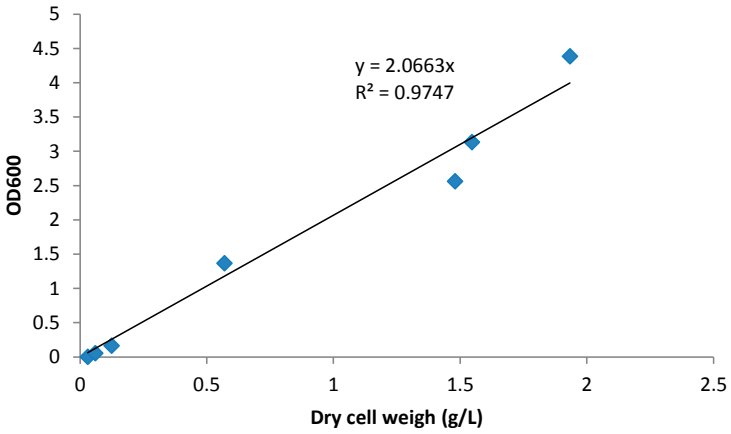

Figure A 3: Fermentation yield obtained from growing cells in LB media

Conversion to dry cell weight

In order to use the cells for biocatalysis, the cells needed to be concentrated. Therefore, the cells from the shake-flasks were harvested (by centrifugation) and resuspended in phosphate buffer to obtain a stock at a concentration of 500 gcww/L. This stock was then diluted to obtain the different cell concentrations. 1 mL of the suspension was filtered and used for dry cell weight measurements.

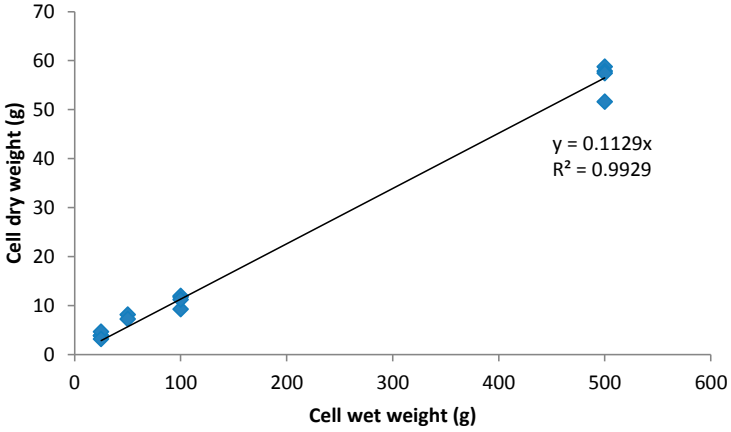

Figure A 4: Conversion of wet cell weight to dry cell weight. Triplicate measurements were made and plotted.

It should be noted that the dry cell weight is about one-tenth of the wet cell weight (Figure A 4). This could be because of the water trapped in the cell pellet which adds to the wet cell weight.

Appendix III – Biocatalyst characteristics

SDS PAGE

In order to check for the protein production in E. coli BL 21 (DE3) strains, SDS PAGE was run (Figure A 5).

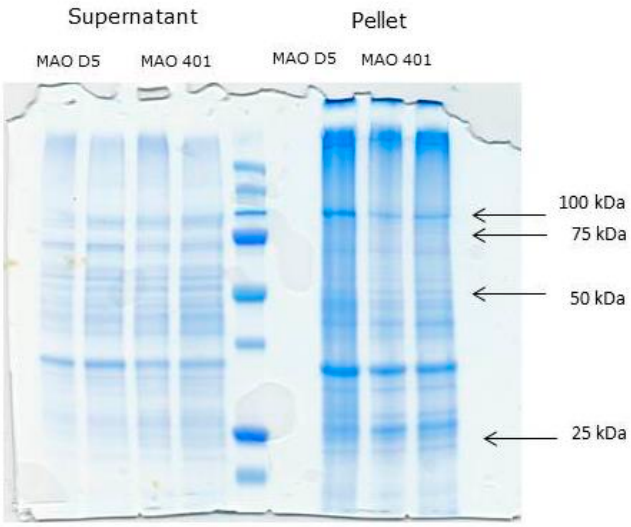

Figure A 5: SDS PAGE of cell pellet (right of molecular marker) and supernatant (left of molecular marker) of lysed E Coli BL21 cells to check for protein.

The molecular weight of the protein is about 55 kDa (Nick Turner, personal communication). The gel indicates that the protein is barely seen around a molecular weight of 55 kDa, indicating that the fermentation yield for the protein is low (expression level).

Test for plasmid leak

To verify if the insert is being lost from the plasmid, we tried to isolate and clone the plasmid onto an HMS strain where we expected better plasmid integrity. After the cloning, both strains were fermented in LB media and used for biocatalysis and the substrate conversion measured and plotted Figure A 6.

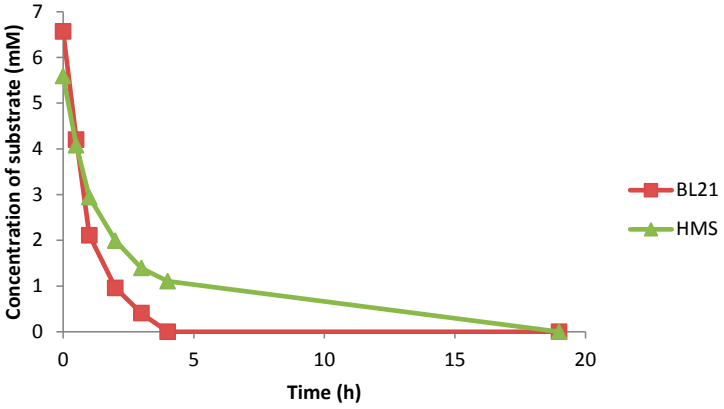

Figure A 6: Comparison of biocatalysis profile when the enzyme was expressed in BL21 and HMS strains.

From the above figure, it can be seen that both the BL21 and HMS strains have very similar reaction profiles indicating no change in shifting from one strain to another.

Then, we decided to check for induction of the protein production using IPTG in the HMS strains to test for effect of induction Figure A 7. 50 µl of 100 mM IPTG was added when the OD_{600} reached a value of 0.9. The test indicated that there is little difference in reaction profiles when induced with IPTG. This indicates that the induction does not help to produce more biocatalyst. This might be due to the protein being constitutive.

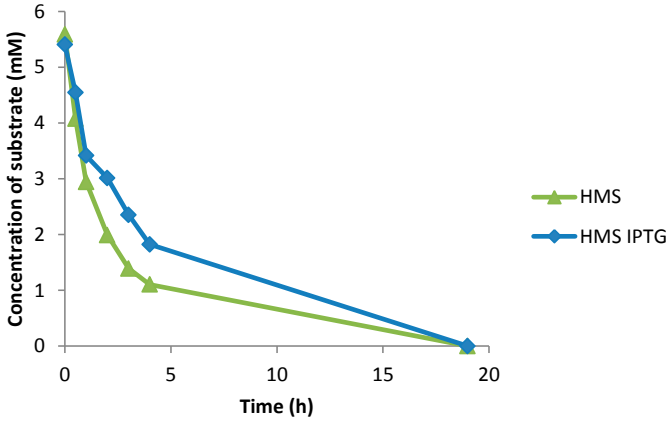

Figure A 7: Test for induction of MAO production using IPTG.

Test for constitutive expression of the protein

In order to check for constitutive expression of the protein, cells were harvested at different times of fermentation and used for carrying out a standard reaction. 1mL of the broth was harvested and the pellet was resuspended in 2mL of phosphate buffer when the cells had been growing for 4h and 6h. In order to get a comparison to the standard reaction the cells harvested after 18h of fermentation (fermentation was carried out without IPTG) was resuspended in phosphate buffer and used for biotransformation at a concentration of 5 gcdw/L. The conversions obtained at these conditions are reported in Table A 4.

Table A 4: Conversions obtained by HMS cells grown for different times and a reaction with 10mM of substrate was carried out and conversion measured at 21h. Duplicates were represented in the table below.

Experiment number	Biocatalyst harvested after fermentation at times t= (h)	Conversion at 21h of biocatalysis (%)
Experiment 1	4	8.07
Experiment 1	4	7.37
Experiment 2	6	14.49
Experiment 2	6	16.30
Experiment 3	24	95.33
Experiment 3	24	92.51

These results indicate that the protein is produced constitutively inside the cells.

Appendix IV – Charge of substrate and product with respect to pH

In order to assess the differences in properties of the substrate and product in terms of charge, a plot of the ratio of uncharged substrate species to the charged substrate and the corresponding pH was plotted (Figure A 8). Plot was obtained using the Hassel-Bach equation and the pKa values of the substrate and the product. The plot indicates that the product is completely uncharged around pH 9.5 while all of the substrate at this pH is charged. This difference in charges can be exploited for product removal strategies.

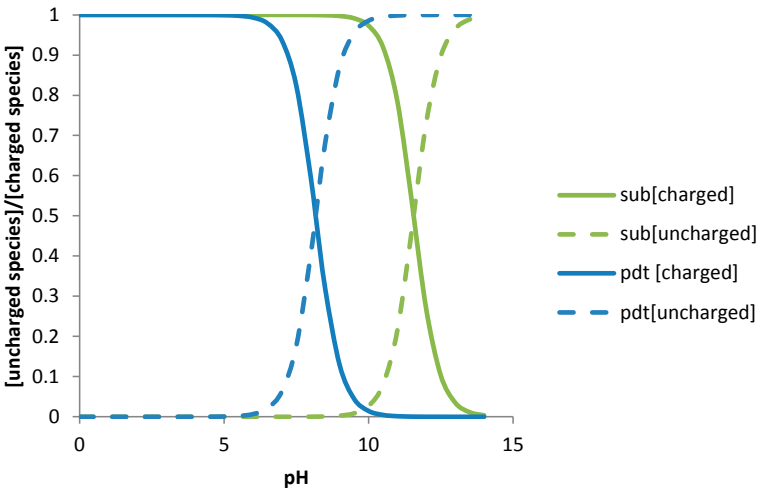

Figure A 8: Effect of pH on the charge of the substrate and the product

Appendix V – Product extraction

In order to obtain product for NMR analysis, three extraction steps with MTBE were made. To determine if the entire product was extracted, the MTBE phase before and after extraction was measured. A plot of the relative response of the product to the number of extraction steps was obtained (Figure A 9). This indicated that the entire product produced during the reaction has been extracted with three steps of extraction.

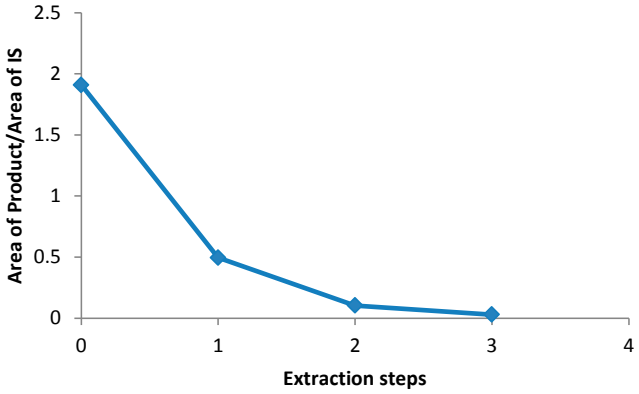

Figure A 9: Extraction of product with MTBE for isolation of product.

Appendix VI – NMR analysis

To confirm the presence of a trimer, NMR analysis was performed. ^{1}H NMR (300 Hz) of the substrate, isolated product and extracts from during the reaction were performed to obtain an idea about the presence of a trimer. NMR obtained was then compared to that from the literature[57].

NMR of Substrate

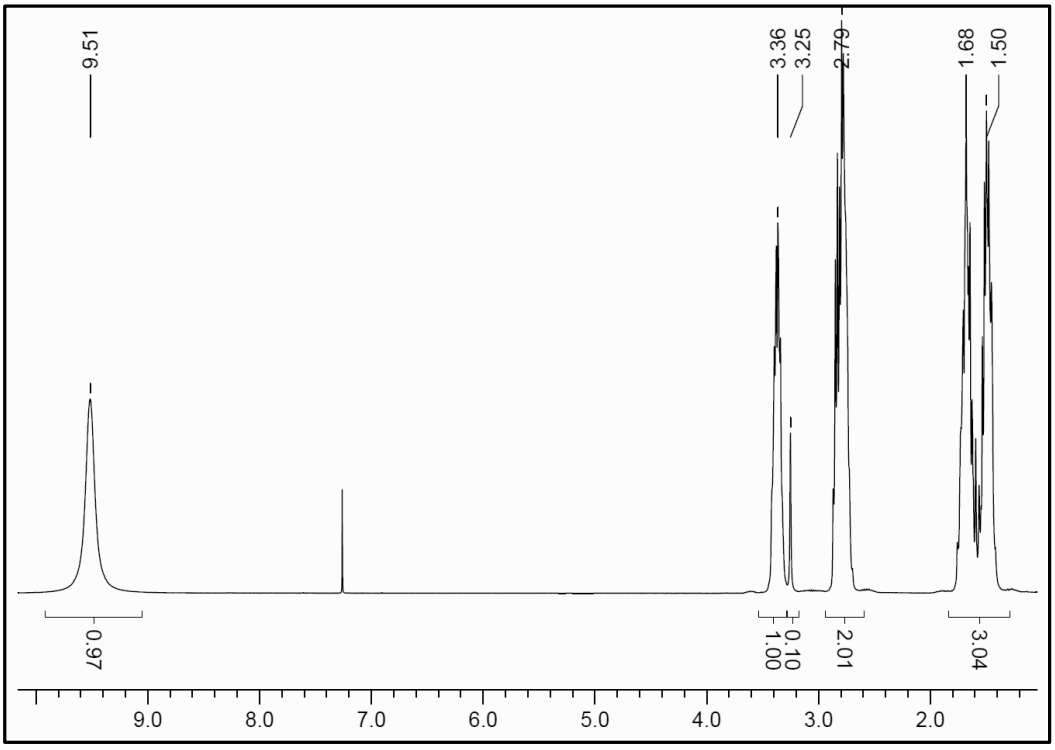

Figure A 10: NMR spectra of pure substrate (3-aza bicycle octane [3,3,0] HCl

NMR of isolated product

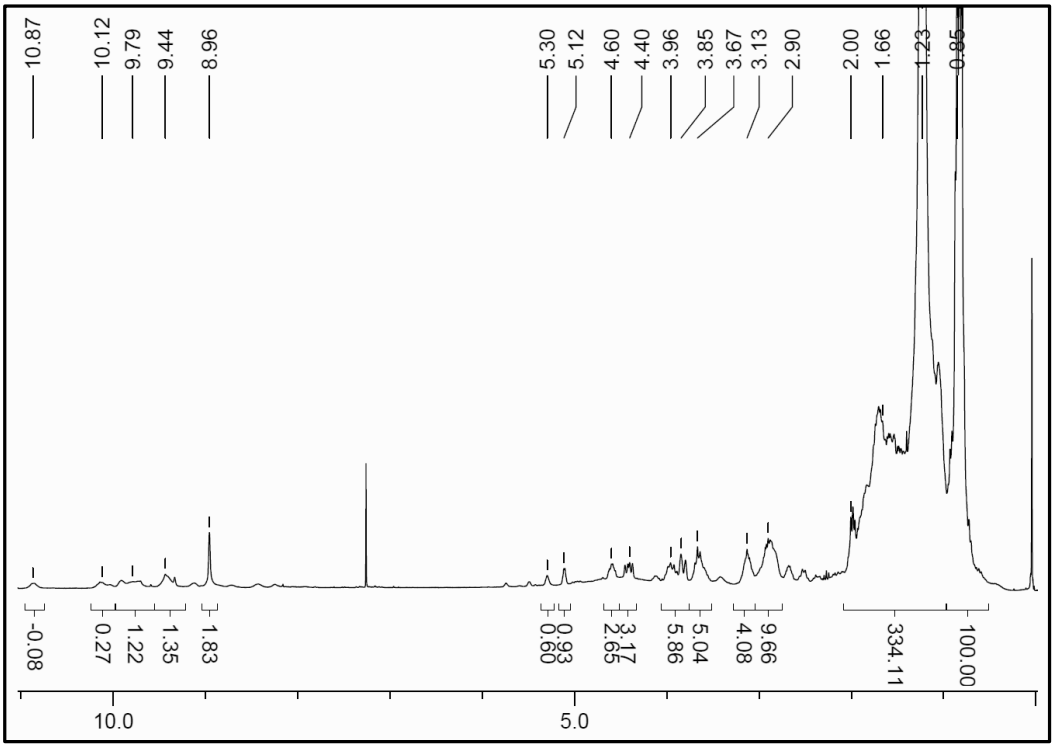

Figure A 11: NMR spectra of isolated product

NMR from biotransformation

The reaction mixture was harvested at t=4h and t=24h.

NMR from biotransformation (t=4h)

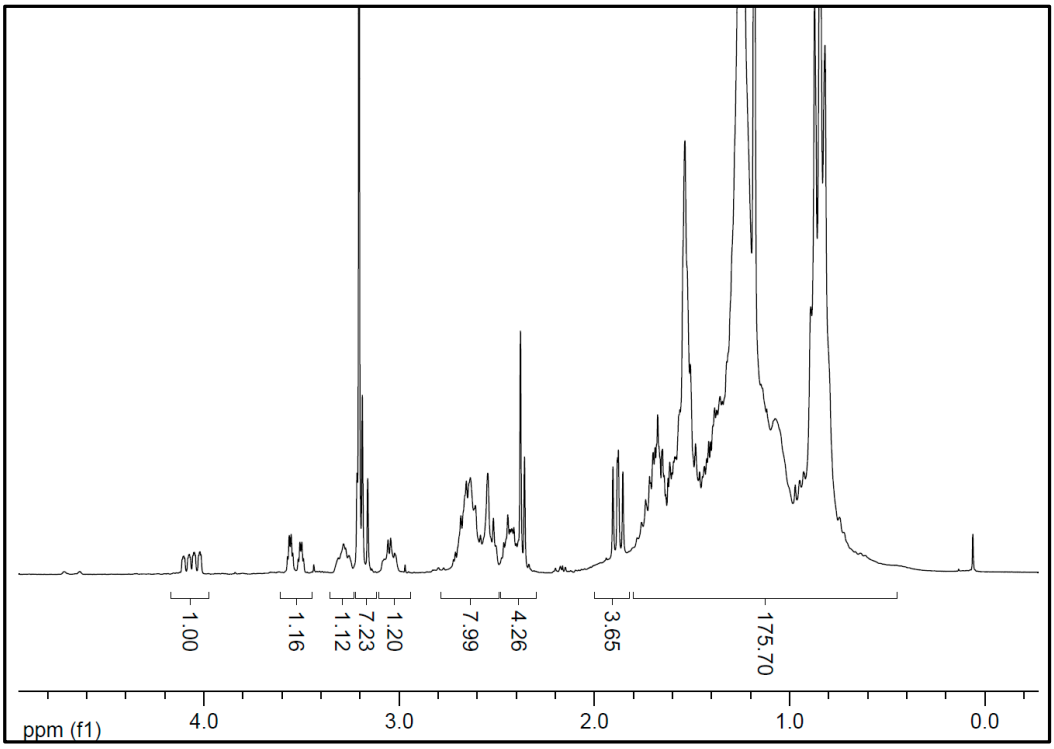

Figure A 12: NMR spectra of biotransformation mixture at t=4h

NMR from biotransformation (t=24h)

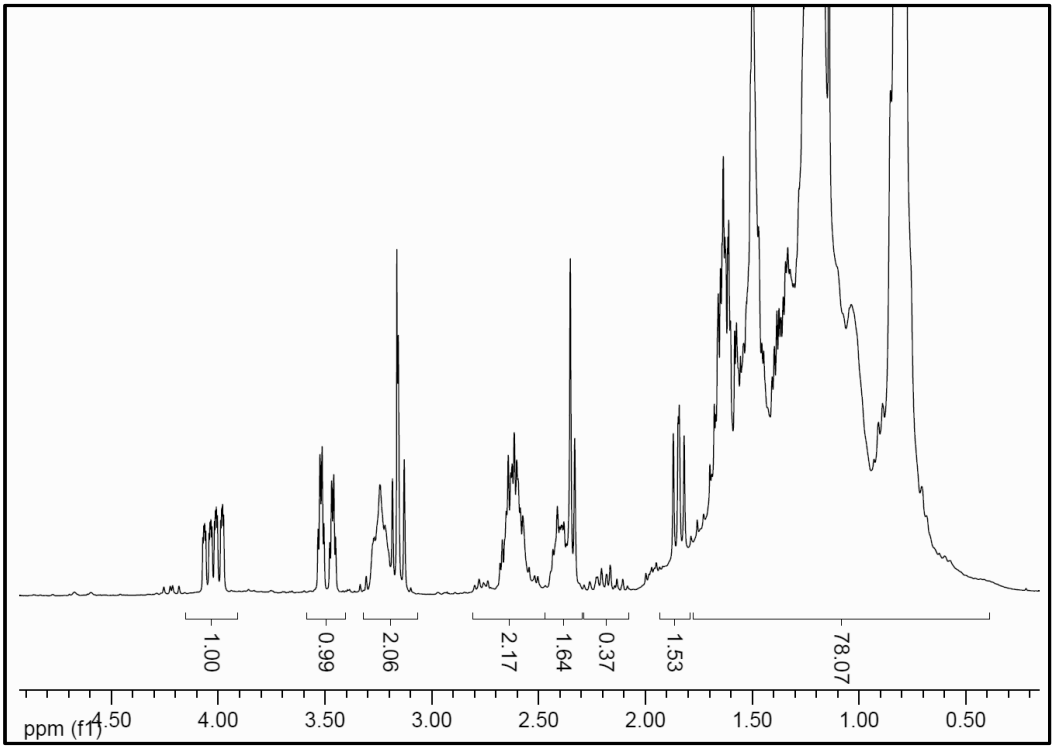

Figure A 13: NMR spectra of biotransformation mixture at t=24h

Appendix VII – Test for rate limitation

To test for rate limitation at different cell concentrations and substrate concentrations, the product concentration obtained was plotted as a function of cell concentration and time. By doing so, the reaction rate is normalised to the biocatalyst concentration. If the reaction system becomes rate limited, the reaction profiles for the different biocatalyst concentrations would not overlap. The figure indicates that the biocatalytic reaction is not oxygen limited at these biocatalyst concentrations.

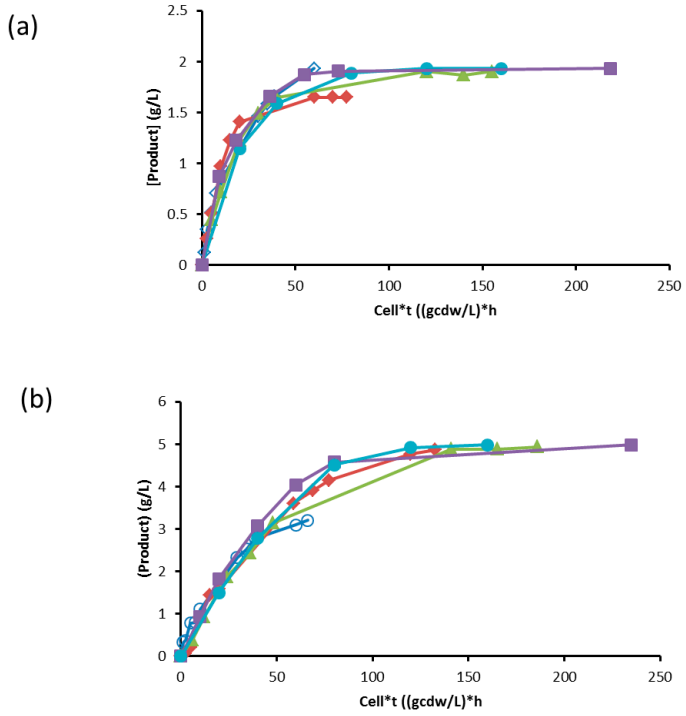

Figure A 14: Product concentration achieved in a reaction where a substrate concentration of (a) 2.6 g/L, (b) 7 g/L was converted as a function of reaction time and biocatalyst concentration. Biocatalyst concentration: (●) – 20 gcdw/L, (■) – 10 gcdw/L, (▲) – 5 gcdw/L, (♦) – 2.5gcdw/L and (○) – 1.25 gcdw/L.

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